

THE ROLE OF LIVER SINUSOIDAL ENDOTHELIAL CELLS IN HEPATITIS C VIRUS INFECTION

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Abstract

Hepatitis C virus (HCV) infection is a major cause of global morbidity, causing chronic liver injury that can progress to cirrhosis and hepatocellular carcinoma. The liver is a large and complex organ containing multiple cell types, including hepatocytes, sinusoidal endothelial cells (LSEC), stellate cells, Kupffer cells and biliary epithelial cells. Hepatocytes are the major reservoir supporting HCV replication, however, the role of non-parenchymal cells in the viral lifecycle remain largely unexplored. Endothelial cell hepatocyte co-cultures were established to study the role of LSEC in HCV biology. Vascular endothelial growth factor (VEGF-A) regulated transcripts were profiled by microarray to identify factors modulating HCV replication.

The initial studies indicated that rather than transmitting HCV to permissive hepatocytes LSEC were protective in HCV infection. Co-culture of epithelial and endothelial cell showed that LSEC limit hepatocyte permissivity to HCV infection via cell contact-dependent mechanisms and by the expression of soluble mediator(s) that are regulated by VEGF-A. Transcript analysis identified LSEC expression of bone morphogenetic protein 4 (BMP4), a novel proviral molecule that is negatively regulated by VEGF-A via a VEGF receptor-2 (VEGFR-2) MAPK dependent pathway. Consistent with the *in vitro* data I observed increased BMP4 expression and reduced VEGFR-2 activation in inflamed liver tissue.

These studies show a novel role for LSEC and BMP4 in HCV infection and highlight BMP4 as a new therapeutic target for treating liver disease.

Dedication

This thesis is dedicated to my wife Liz, my sons Benjamin and Rupert, and to my parents, Steve and Sally, who gave me the opportunities to excel in my chosen career.

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Frequently used abbreviations

BMP	bone morphogenetic protein
BSA	bovine serum albumin
CHO	Chinese hamster ovary
CM	conditioned medium
FGF	fibroblast growth factor
HCV	hepatitis C virus
HGF	hepatocyte growth factor
IgG	immunoglobulin G
IL	interleukin
LPS	lipopolysaccharide
LSEC	liver sinusoidal endothelial cells
NS	non-structural
PBS	phosphate buffered saline
PIGF	placental growth factor
SR-BI	scavenger receptor BI
TGFβ	transforming growth factor β
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

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Chapter 1 Introduction

1.1 General introduction

The liver is a large and complex organ that has fundamental roles both in the development and normal function of vertebrate organisms. These functions of the liver are frequently taken for granted but the occurrence of liver failure provides a daily insight for physicians caring for patients with this condition. Although acute liver failure is rare it provides an illustration of the functions of the liver, namely gluconeogenesis, clearance of toxic metabolites, conjugation of bile pigments and drugs, and synthesis of plasma proteins.

Chronic liver disease has by definition a more insidious onset and is characterised by fatigue and malaise in its early stages. Repetitive liver injury of any cause, most commonly alcohol, but also fatty liver, autoimmunity, and chronic viral infection establishes chronic inflammation in the liver. As a consequence of on-going injury and inflammation there is a wound healing response that is characterised by the generation of fibrosis. An accumulation of fibrosis in liver disease is common and when severe is termed cirrhosis. As disease becomes more advanced and cirrhosis is established, features of liver failure develop, most frequently jaundice and fluid

retention. Catastrophic bleeding events and infections are also common. Cirrhosis itself is also a risk factor for the development of primary liver cancer (hepatocellular carcinoma, HCC). At this stage of disease there is no medical treatment that is effective and rather liver transplantation is considered. Transplantation is an imperfect treatment and improving medical treatments for patients with liver disease is likely to reduce the need for transplantation in the future. Moreover due to the inherent limitations of transplantation and the availability of donor organs many patients die from liver disease. Most strikingly, 1 in 9 deaths in individuals aged between 40 and 49 is due to liver disease (**Fig. 1-1**). Furthermore liver disease is, in contrast to heart disease, stroke, and cancer, the only major cause of death that continues to increase in prevalence. Thus there are many major unmet needs for patients with liver disease that if adequately addressed would benefit patients and society.

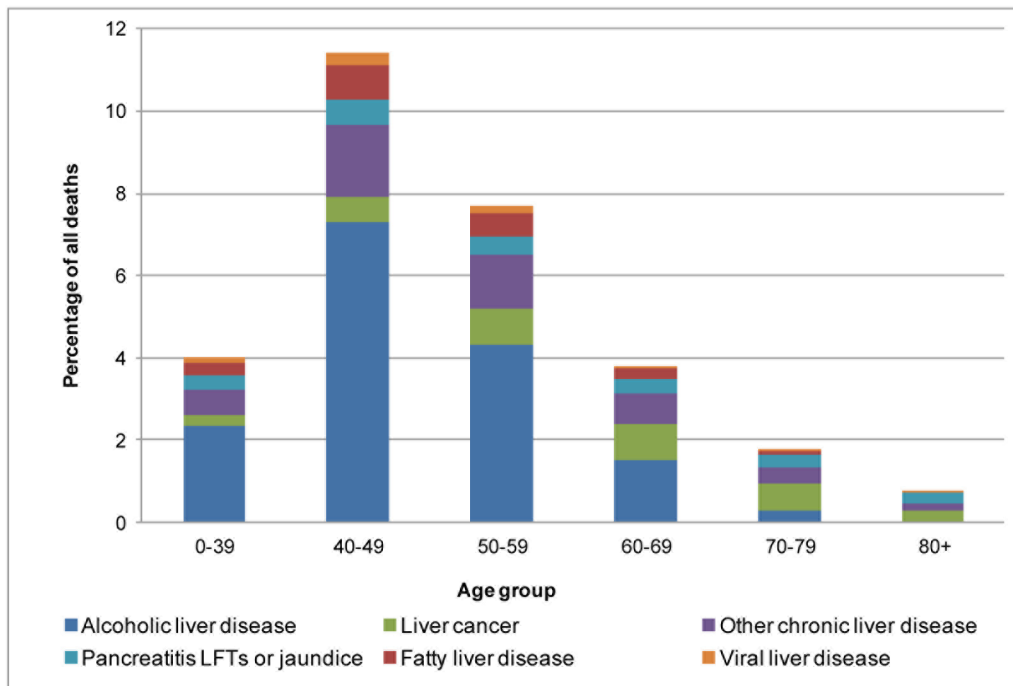


Figure 1-1. The proportion of deaths attributable to liver disease by age.

The proportion of deaths in England attributed to liver disease as recorded by the Office for National Statistics (National End of Life Care Intelligence Network, 2012).

Hepatitis C virus (HCV) infection is common (Shepard et al., 2005), and is a leading reason for liver transplantation in the UK and the rest of the western world (Brown, 2005). Until recently the only available treatment for this chronic viral infection was combination therapy with pegylated interferon- α and ribavirin, a non-specific antiviral drug with uncertain mechanism of action. The last year has seen the licensing of the first class of specific targeted antiviral therapies for HCV. These have increased response rates but come at the expense of increased adverse effects since they are given in combination with interferon and ribavirin as triple therapy (Rowe and Mutimer, 2011). Many other specific therapies are in development that will further increase response rates and likely reduce both the frequency and severity of adverse events (Poordad and Dieterich, 2012). These developments are welcome and will, if treatment is widely used, change the outlook for many patients with HCV (Rowe et al., 2012). The next great need in this disease is the identification of therapeutic targets that modulate established fibrosis, or cirrhosis, or that decrease the risk of cancer that remains even after viral eradication. These strategies would likely also be applicable for patients with liver diseases of all aetiologies since fibrosis progression and development of liver cancer is a common pathway in liver disease.

A detailed understanding of the pathogenesis of liver injury that is mediated by HCV is one path to uncovering novel therapeutic targets in liver disease. In this thesis aspects of the viral lifecycle and the influence of “bystander” endothelial cells in the

liver will be discussed. Ultimately a novel pathway active in liver disease that HCV may have adapted to exploit will be described.

1.2 Anatomy and function of the liver

Gross anatomy

The liver is the largest solid organ in the body and weighs between 1200 and 1500g. It performs many varied functions that are required for survival and without which death ensues rapidly. Hidden from the trauma of everyday life by the ribs on the right side of the abdomen, anatomically the liver is divided into two lobes. The right lobe is much larger than the left and these are separated by the falciform ligament (actually a fold of peritoneum) anteriorly, and by the ligamentum teres inferiorly. These structures act as physical support for the liver that, together with intra-abdominal pressure from the normal tone of the abdominal wall, holds the liver in position.

The liver is perfused with a dual blood supply. 80% of inflow comes directly from the gut in portal circulation; the remaining 20% of supply is from the hepatic artery that brings freshly oxygenated blood from the heart via the coeliac axis. Both vessels enter the liver at the porta hepatis before dividing into branches and being distributed by the sinusoidal networks. This mixed blood is then collected in the central vein where drainage is subsequently into the hepatic veins and ultimately into the inferior vena cava just inferior to the entry to the right atrium. Understanding of this vascular supply has allowed functional classification of liver

anatomy. This classification described by Couinaud (Couinaud, 1957) defines 8 segments (**Fig. 1-2**) that are functionally distinct since there are no macroscopic vascular anastomoses (although blood may mix at the sinusoidal level). The classification allows for rational surgical treatment planning when considering resection of hepatic lesions, including primary liver cancers that are associated with chronic liver disease (Bismuth, 1982).

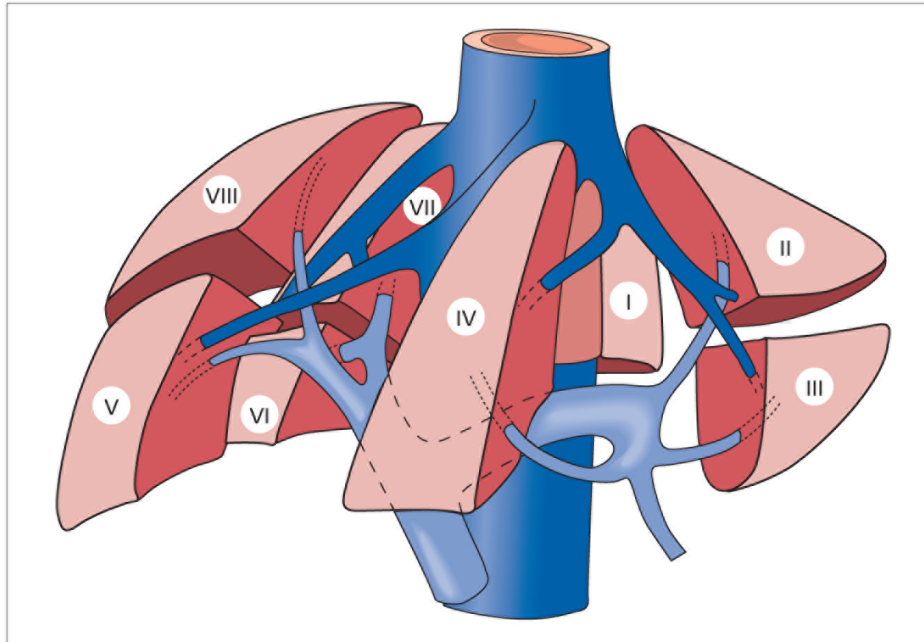


Figure 1-2. The functional anatomy of the liver.

The blood supply from the portal vein branches are shown in light blue and the drainage into the hepatic veins is indicated in dark blue (Lefkowitz, 2011).

Liver ultrastructure

The major cell type populating the liver is the hepatocyte. These cells occupy approximately 80% of liver volume (Blouin et al., 1977) and are responsible for many of the essential functions of the liver. These parenchymal cells have multiple and complex interactions with non-parenchymal cells, namely liver sinusoidal endothelial cells (LSEC), biliary epithelial cells, Kupffer cells (liver resident macrophages), and hepatic stellate cells. The hepatocytes form single cell thick chords bound together by tight junction proteins. These separate the apical surface that forms the border of the canalicular structures from the basolateral surface that faces the sinusoidal lumen (**Fig. 1-3A**). The canaliculi resulting from this cellular polarity are responsible for draining bile (the major exocrine product of the liver) into the bile ducts that are located in a triad with the hepatic artery and portal vein. These triads are arranged in a hexagonal distribution, each hexagon surrounding a central vein. Blood entering these hexagonal structures, termed lobules thus flows from the portal vein and hepatic artery inflow to the central vein and bile is collected in the opposite direction (**Fig. 1-3B**).

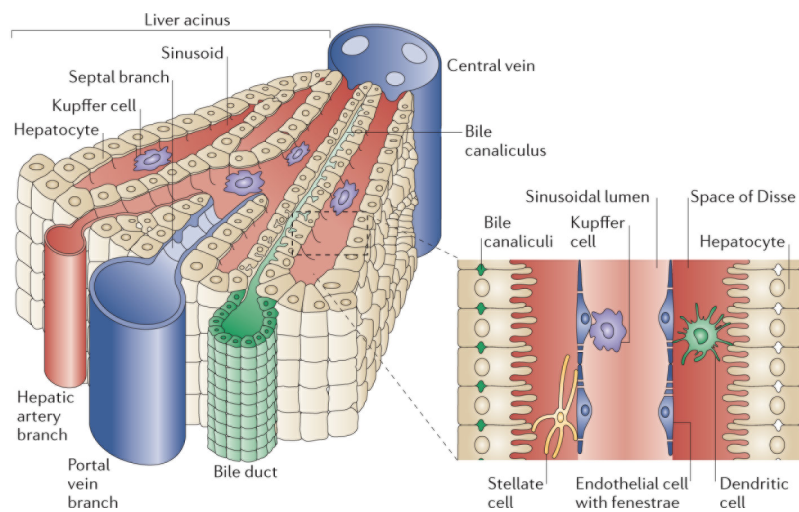
A**B**

Figure 1-3. The liver architecture.

False colour scanning electron micrograph of a freeze fracture through mouse liver. Hepatocytes are coloured green, and liver sinusoidal endothelial cells are coloured pink. Canalicular structures are seen running through the chords of hepatocytes. Image courtesy of the Wellcome Trust image library (A). Schematic diagram of the liver lobule or acinus (Adams and Eksteen, 2006) (B).

There are important differences in hepatocellular function between hepatocytes depending on their location in the liver lobule. Those positioned closest to the portal triad – zone one – have different functions to those positioned adjacent to the central veins – zone three. These differences are related to differences in lobular oxygenation (Jungermann and Kietzmann, 2000), with higher oxygen delivery to zone one than to zone 3, and, as has recently been described, are also dependent on signals relayed by the Wnt/ β catenin system (Burke et al., 2009). For instance drug metabolising cytochrome P450 enzymes are more abundant in zone three. These hepatocytes also have reduced glutathione concentration rendering these cells more susceptible to drug related injury. Indeed these drug reactions are typified by centrilobular hepatocyte injury when liver biopsies are taken from affected patients. Similarly the commonly used mouse model of carbon tetrachloride causes a similar injury for the same reasons (Friedman, 2010).

The understanding of the development of this complex arrangement of multiple cell types is critical in understanding the response of the organ to liver injury. The molecular cues that are required during development are also likely to be activated in the regenerating liver after partial liver resection.

1.3 Development of the liver

Due to the size of the liver and the relative accessibility of tissue samples the development of the liver is well characterised (Lemaigre, 2009, Si-Tayeb et al., 2010). Initial formation of the liver bud, and specification of primitive hepatoblasts that go on to form both hepatocytes and biliary epithelial cells occurs early in development and requires multiple signals that are co-ordinated in both space and time.

Hepatoblasts have their origins initially in the definitive endoderm that is established during gastrulation. This lies in close proximity to the developing heart that provides the first instructive signals in specification to a hepatoblast fate. These signals were described to be members of the fibroblast growth factor (FGF) family (Deutsch et al., 2001). Although many FGF family members are expressed in the developing heart there is significant redundancy since genetic manipulation of each of these factors has little effect (Miller et al., 2000). Following initial specification a second signal is required from the developing septum transversum mesenchyme. Bone morphogenetic protein 4 (BMP4) is highly expressed in the septum transversum mesenchymal cells, as is its regulator the transcription factor GATA4 (Nemer and Nemer, 2003). Genetic deletion of either of these factors significantly delays both hepatoblast specification, and also the early migration of the liver bud into the septum transversum mesenchyme (Watt et al., 2007, Rossi et al., 2001). Interestingly both GATA4 and BMP4 are expressed in other cell types at the time of

specification but it is the signals derived from the septum transversum mesenchyme that are critical for liver development. These findings are supported by the findings that both GATA4 and BMP4 are indispensable for liver bud formation in zebrafish (Shin et al., 2007).

Following specification there is a proliferation of hepatoblasts as they bud into the adjacent stroma. This process is related to the provision of stimulatory signals from endothelial cells within the stroma (Lammert et al., 2001, Lammert et al., 2003, Matsumoto et al., 2001). The soluble signals that are secreted by endothelial cells have not been identified to date. However it is interesting that liver endothelial cells have been reported to drive hepatocyte proliferation after liver resection in mouse models through expression of hepatocyte growth factor and other factors (LeCouter et al., 2003, Ding et al., 2010). This migration into the stroma and subsequent formation of the liver bud is associated with a change in transcriptional networks in the hepatoblasts. There is expression of the homeobox transcription factors *Hhex*, and *Prox1*, as well as *Hnf6*, and *OC-2* (Zaret and Grompe, 2008).

The on-going development of the liver is supported by the generation of the sinusoidal networks that distribute blood across the liver. The sinusoidal endothelial cells develop from the endothelial cells within the septum transversum mesenchyme (Gouysse et al., 2002). As development progresses these endothelial cells differentiate into the mature sinusoidal endothelial cell phenotype. The signals

that are required for this process are not well established but it is apparent that there are contemporaneous changes in the supporting matrix suggesting that such cues may be important (Couvelard et al., 1996). Another possibility is a role for Wnt signalling (Zeng et al., 2007, Klein et al., 2008, Matsumoto et al., 2008): Wnt2 has been shown to increase the proliferation of rat sinusoidal endothelial cells, and that when Wnt2 levels were depleted there was a reduction in the expression of vascular endothelial growth factor receptor 2 (VEGFR-2) (Klein et al., 2008) that is responsible for mediating the mitogenic activity of that growth factor (Quinn et al., 1993, Olsson et al., 2006).

The final important step in establishing the liver lobule is in the development of hepatocellular polarity. In chick embryo studies the first signs of polarity – the detection of apically expressed proteins – occurs soon after the development of the liver bud, but fully mature structures are not seen until after birth (Gallin and Sanders, 1992). The mediators implicated in the development of polarity have largely been extrapolated from other polarised cell systems (Nelson, 2003, Wang and Boyer, 2004) however two mechanisms that pertain to hepatocyte polarity have been identified in human cell systems. Firstly, signals through the interleukin (IL) 6 receptor unit gp130 drives bile canalicular formation in the hepatoma cell line HepG2 (van der Wouden et al., 2002). Oncostatin M, an IL6 related cytokine, is also implicated in the early stages of liver development, including in the differentiation of hepatoblasts to hepatocytes (Kamiya et al., 1999). Secondly, the extracellular matrix

component fibronectin has also been shown to stimulate the development of hepatocellular polarity (Herrema et al., 2006). Interestingly, given the close relationships between developing hepatocytes and endothelial cells in the nascent sinusoids a direct cellular interaction has been described in zebrafish (Sakaguchi et al., 2008). Using previously identified mutants a role for endothelial cell-cell junction proteins *valentine* (human homolog, cerebral cavernous malformation (CCM) 2), and *heart of glass* (human homolog HEG1) a binding partner for *valentine* was identified as regulators of hepatocellular polarity. Incorporation of cells overexpressing *heart of glass* modified the polarisation of surrounding cells suggesting direct regulation by this protein in the liver. These studies indicate that the regulation of hepatocellular polarity is complex requiring multiple signals that have likely not been completely elucidated.

A fundamental role for endothelial cells is therefore established at several stages of development of the liver suggesting that these cells are critical in the organ's response to injury.

1.4 Liver sinusoidal endothelial cell biology

Liver sinusoidal endothelial cells (LSEC) provide the physical distribution system for blood entering the hepatic lobule. Whilst hepatocytes make up the majority of liver volume LSEC comprise some 40% of the remaining non-parenchymal cells, or 3% of the total liver volume (Blouin et al., 1977). The initial identification of LSEC as a unique microvascular cell type dates from the development of high resolution electron microscopy techniques. Indeed the first description of LSEC fenestration, the characteristic feature of this cell type, was from Wisse in 1970 (Wisse, 1970). Since that time the study of LSEC has expanded from those investigators interested in pure endothelial cell biology to investigators interested in immunology, pharmacology, virology, and the contribution of endothelium to metabolism.

Ultrastructural characteristics of LSEC in vivo

The identity of LSEC remains a controversial topic. *In vivo* LSEC are best characterised by the presence of fenestrations. These fenestrations are organised into sieve plates that are critical for normal endothelial cell function. In human LSEC the endothelial fenestrae measure 150-175nm in diameter and occur at a frequency of 9-13 per μm^2 . Thus these fenestrae occupy approximately 7% of the endothelial cell surface (Wisse et al., 1985) (**Fig. 1-4**). Further important ultrastructural

characteristics that are functionally significant are the presence of numerous micropinocytic vesicles and many lysosome-like structures (Braet and Wisse, 2002).

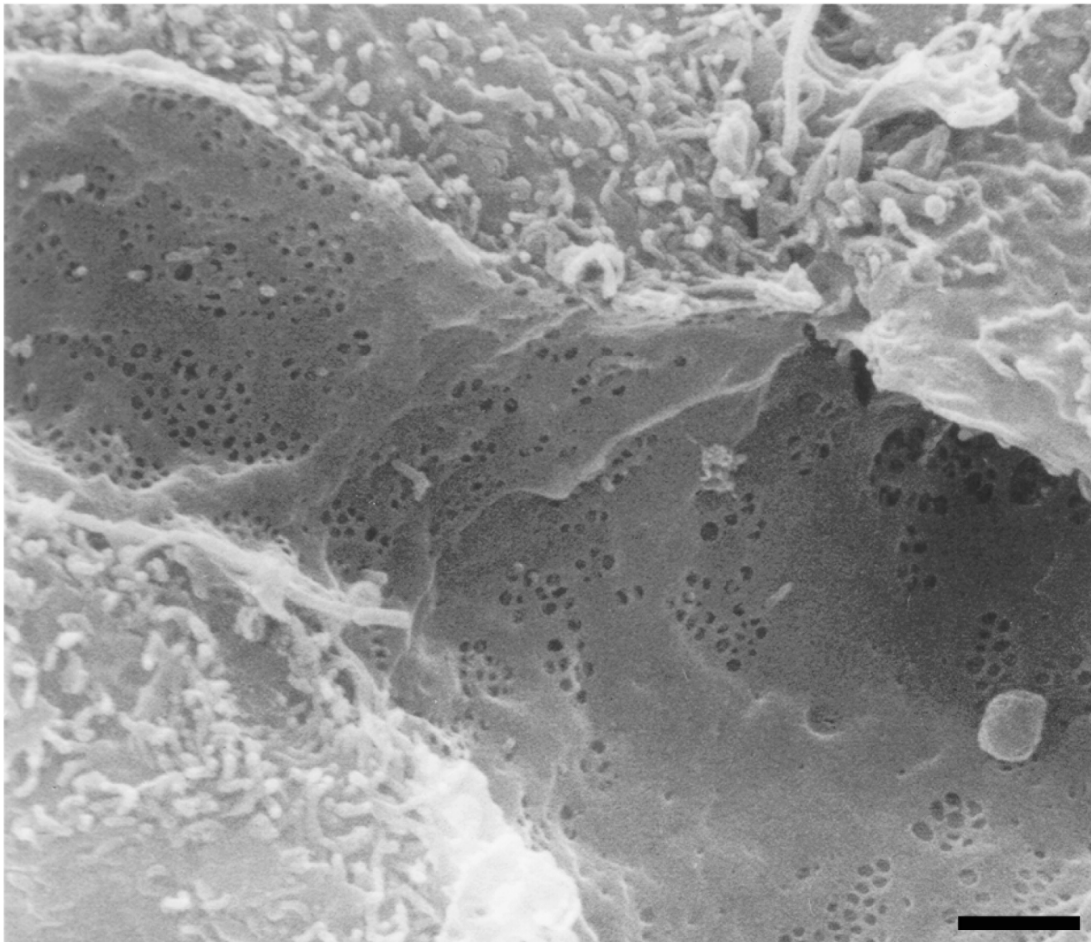


Figure 1-4 Sinusoidal endothelial cell fenestrations are organised into sieve plates.

Scanning electron micrograph of rat liver highlighting endothelial cell fenestrations. Scale bar, 1 μ m (Braet and Wisse, 2002).

These ultrastructural findings indicated the prominent roles of LSEC *in vivo*. Firstly the organisation of sieve plates suggested the function of LSEC as a dynamic filter for substances absorbed from the gut and delivered to the liver in the portal circulation. Secondly the presence of multiple endocytic vesicles indicated the high endocytic capacity LSEC and a critical functional role in clearing waste macromolecules from the circulation.

It is important to note that these ultrastructural studies also indicated the presence of only a minimal basement membrane supporting LSEC in the normal liver (Braet and Wisse, 2002). This finding is in keeping with the functions of LSEC suggested above since the absence of a significant quantity of basement will allow the free passage of substances through the dynamic filter of the sieve plates.

Molecular characteristics of LSEC

To further dissect the function of LSEC it was important to reliably identify cells isolated from whole liver. However a number of different markers have been used for this purpose that are not consistent between species, or indeed between investigators. *In vivo*, LSEC express a different repertoire of surface markers to other microvascular and large vessel endothelia. For instance LSEC are characteristically CD31 low and expression is typically intracellular in normal conditions, in contrast vascular endothelium shows high level CD31 expression (Lalor et al., 2006).

The specialised function of LSEC suggests the presence of specific phenotypic protein expression. Indeed several receptors expressed by LSEC have been suggested as phenotypic markers of this cell type (Lalor et al., 2006). These receptors relate specifically to scavenger function and interestingly often show overlapping expression with lymph node endothelium. Those receptors characterised to have predominant LSEC (and lymph node) expression include stabilin-1, and -2 (Hansen et al., 2005, Politz et al., 2002); liver/lymph node specific ICAM-3-grabbing nonintegrin (L-SIGN) (Bashirova et al., 2001) and the related molecule liver and lymph node sinusoidal endothelial cell c-type lectin (LSECtin) (Liu et al., 2004); and lymphatic vessel endothelial hyaluronan receptor (LYVE-1) (Mouta Carreira et al., 2001). These receptors have an important role in the scavenger functions of LSEC, and provide an important insight into the phenotypic characterisation of isolated cells *in vitro*.

Functional characteristics of LSEC

The phenotypic characteristics of LSEC have, as described, given major clues as to the identity of the functions of LSEC. Broadly these have been characterised into filtration and scavenger activity. However it is now recognised that LSEC have additional functions that relate to immune function of the liver and the tolerance to repetitive immune stimulation by bacterial products carried in portal blood (Knolle and Gerken, 2000, Crispe, 2009).

Filtration functions

The role of fenestrations in the “liver sieve” has been carefully elucidated largely using lipoprotein components. These range in size from 50-100nm in diameter for chylomicron remnants to 100-1000nm in diameter for intact chylomicrons. Isolated hepatocytes are unable to distinguish between these lipoproteins *in vitro* (Floren, 1984), but in the liver only the chylomicron remnants are taken up by hepatocytes. Furthermore, intact chylomicrons were identified only in the sinusoidal lumen whereas chylomicron remnants were identified in the space of Disse, the anatomical space between the sinusoidal endothelium and underlying hepatocytes (Fraser et al., 1978). These findings are further supported by additional studies with molecules of differing physical properties. For example nonliposomal doxorubicin (a chemotherapeutic agent) is taken up by hepatocytes whereas the liposomal formulation is excluded from the space of Disse (Hilmer et al., 2004). It is noteworthy that this change in pharmacokinetics leads to an increase in systemic drug exposure of the liposomal compound of >300-fold, and a decrease in clearance of >250-fold highlighting the importance of the action of the liver sieve in hepatic drug clearance. These findings support a model where small particles <100nm in diameter readily pass through LSEC fenestrations in a sieving process.

Scavenger functions

The scavenger functions of LSEC have been well defined in studies using rats using an array of macromolecules and other potentially hazardous components of

connective tissue. The endocytic receptors in rat have been defined in five functional groups:

1. Collagen α -chain receptor (COLLAR) has specificity for free α -chains of type I to V, and IX collagen but not native triple helical collagen and this receptor has been identified exclusively in LSEC (Smedsrod et al., 1985a).
2. Hyaluronan receptor (HAR) is responsible for clearance of circulating hyaluronan (Eriksson et al., 1983). This receptor that has been purified and characterised to be a high affinity receptor for hyaluronan (McCourt et al., 1999), as well as a number of other ligands including chondroitin (Smedsrod et al., 1985b). The receptor efficiently clears hyaluronan from the plasma. Hyaluronan has a plasma half-life of less than one minute and large amounts of protein (10-100mg in humans) are cleared from the plasma each day (Engstrom-Laurent and Hallgren, 1985).
3. The immunoglobulin G Fc receptor (that is expressed on both LSEC and Kupffer cells) has been shown to eliminate circulating immune complexes from the bloodstream (Lovdal et al., 2000).
4. Mannose receptor activity has been shown to be extremely rapid, indeed the cell surface half life of the receptor is estimated at 10 seconds (Magnusson and Berg, 1989). The receptor recognises the terminal non-reducing sugar residue of the oligosaccharide (fucose, mannose or N-acetyl-glucosamine) moiety of glycoproteins (Ashwell and Harford, 1982). Interestingly the mannose receptor has also been implicated in the sorting of antigens for cross presentation to CD8 T cells (Burgdorf et al., 2007), a pathway that has

been identified as a mechanism of tolerance development in the liver (Diehl et al., 2008).

5. The scavenger receptor family is a growing group of receptors that clears a larger number of macromolecules from circulation including glycosaminoglycans, hyaluronic acid and chondroitin (Harris et al., 2007). The most well characterised human receptors in this family are stabilin-1 (also termed CLEVER-1 and FEEL-1), and stabilin-2 (FEEL-2) (Hansen et al., 2005, Politz et al., 2002). The functional significance of these receptors is highlighted by the recent report of glomerulofibrotic injury in mice lacking expression of both stabilin-1, and stabilin-2 (Schledzewski et al., 2011). Mechanistically this fibrotic injury was related to failed clearance of transforming growth factor β (TGF- β) family member growth differentiation factor 15 (GDF-15). This finding underscores the importance of scavenger activity for the function of the whole organism, and not just in liver function.

Immune functions

The liver is a tolerogenic organ (Crispe, 2009, Protzer et al., 2012). When considering normal liver function, tolerance is expected. Multiple antigens, including food antigens, microbial products, and those from injured gut epithelial cells are constantly delivered to the liver in portal blood. Continuous immune responses to these antigens would be deleterious and therefore mechanisms that dampen immune responses in the liver have developed. Two particular tolerogenic

mechanisms involve LSEC directly. The first of these relates to tolerance to bacterial products. Lipopolysaccharide (LPS) is a common component of bacterial cell walls and this potently stimulates immune responses through activation of the pattern recognition receptor toll-like receptor 4 (TLR4). However, proinflammatory bacterial products including LPS are constantly delivered to the liver and are cleared in the absence of an inflammatory response. Studies of isolated murine LSEC indicated that repetitive stimulation of these cells with LPS rapidly lead to the development of a refractory state where activation of signalling downstream of TLR4 was not sustained but, importantly, clearance was maintained (Uhrig et al., 2005). The second of these relates to the unusual antigen presentation function of LSEC (Knolle et al., 1998, Lohse et al., 1996). Many cell populations in the liver have the ability to present antigen and in the case of LSEC this usually results in tolerogenic activation of T cells (Diehl et al., 2008, Knolle et al., 1998, Knolle et al., 1999, von Oppen et al., 2008). Indeed this tolerogenic activation may be one reason why the liver is such fertile ground for chronic viral infection (Protzer et al., 2012).

1.5 Regulation of LSEC phenotype

The many and varied distinct functions of LSEC suggest the need for maintenance signals that are not required by other endothelial cells. In keeping with this hypothesis LSEC dedifferentiate when cultured *in vitro* as evidenced by a rapid loss of fenestrations and sieve plates (DeLeve et al., 2004, Elvevold et al., 2008). *In vitro* studies have been hampered by the lack of an agreed well-defined molecule that defines normal sinusoidal endothelial cell phenotype. Indeed the phenotypic markers described above have not been studied in relation to regulation of LSEC phenotype.

Regulation of endothelial cell function in general has been variously described to be dependent on interactions with paracrine signalling systems, heterotypic cell types, and basement membrane components. In the case of LSEC, *in vivo* these cells are in close proximity to hepatocytes as well as hepatic stellate cells that are, amongst many other functions, considered to be the pericyte in the liver. It is likely therefore that these cell types provide signals to LSEC to maintain fenestration, and other aspects of normal cellular function. Co-cultivation of hepatocytes with LSEC *in vitro* has been associated with the maintenance of a microvascular phenotype although initially the mechanism defining this process was not well understood (Modis and Martinez-Hernandez, 1991). It was shortly after this report that alterations in vascular endothelial growth factor (VEGF) signalling were noted to have marked

effects on LSEC function *in vitro* (Yamane et al., 1994, Mochida et al., 1998). Furthermore VEGF-/- mice show delayed liver development and an abnormal sinusoidal network further illustrating the importance of this growth factor in sinusoidal endothelial cell biology (Gerber et al., 1999). Taken together these studies highlighted a likely role for VEGF-A signalling in the maintenance of LSEC phenotype.

A clear role for VEGF-A signalling in the normal liver has been further defined both *in vivo*, and *in vitro*. VEGF-A expression in the liver is detectable in hepatocytes but not in LSEC (Yamane et al., 1994, Maharaj et al., 2006). LSEC however express the cognate receptors for this growth factor whereas expression of the receptors is undetectable on hepatocytes (Yamane et al., 1994, Ding et al., 2010, Cao et al., 2010). This pattern of ligand receptor expression indicates the likely presence of a paracrine signalling system involving the closely opposed hepatocytes and sinusoidal endothelial cells of the lobule.

The defining role of VEGF-A regulation of LSEC function was provided by an elegant *in vivo* study from Carpenter and colleagues. These investigators used a model where a VEGF-A trap molecule was expressed specifically in hepatocytes using an inducible system controlled by administration of tetracycline (Carpenter et al., 2005). This trap was able to deplete VEGF-A expressed by hepatocytes thus preventing activation of adjacent endothelial cells. Furthermore, since the system was

inducible it was possible to investigate the role of VEGF-A during development, and during normal tissue homeostasis in the adult liver. The prior studies in VEGF-/- knockout animals had indicated that VEGF-A signalling was required for normal development of the liver (Gerber et al., 1999) but using the inducible system it was apparent that VEGF stimulation was required throughout liver development for normal formation of sinusoidal networks. Further it was noted that there was limited lipid accumulation in the livers of tetracycline treated animals. Given the understanding of LSEC fenestration and its role on accumulation of lipoprotein remnant the authors then went on to study fenestrations in adult mice where VEGF signalling had been inhibited in the post-natal period. These mice also displayed reduced lipoprotein remnant uptake and electron microscopy studies of the liver showed significantly reduced LSEC fenestration. Thus *in vivo* VEGF is required both for the normal development of sinusoidal networks, and for the maintenance of the normal fenestrated LSEC phenotype. These findings have been further supported by the detection of abnormalities in mice with an endothelial specific conditional deletion of VEGFR-2 (Sison et al., 2010).

In vitro evidence of the importance of VEGF-A stimulation comes from studies of rat LSEC immediately following isolation (DeLeve et al., 2004). In these experiments endothelial cell fenestration, and localisation of CD31 was monitored over the following 72 hours. In the presence of control medium there was rapid loss of fenestration and a change in localisation of CD31 from intracellular to membrane

bound. Interestingly these changes were reversed by incubation of LSEC with hepatocyte conditioned medium. The authors hypothesised that this was due to the presence of VEGF in the conditioned media and confirmed this by treatment of conditioned media with anti-VEGF neutralising antibody. The function of VEGF-A in LSEC was described to be mediated by the production of nitric oxide (NO), a well-established downstream target of VEGF-A stimulation (Fulton et al., 1999).

1.6 Vascular endothelial growth factor (VEGF) biology

During evolution vascular networks have developed to permit increasing complexity of organisms, so that oxygen and nutrients can be delivered efficiently to the areas of need. Further these vessels allow immune cells to patrol for the purpose of immune surveillance. Whilst vascular structures allow development and functioning of these organisms they also permit the development of inflammatory and malignant disease by provision of nutrients for these processes. It was the association with malignant disease that cast the light on the development of blood vessels since it was hypothesised that targeting this process would prevent tumour growth and metastasis (Folkman et al., 1971, Folkman, 1971).

Vascular endothelial growth factors (VEGFs) are the foremost signals (amongst many) that regulate the complex processes of blood vessel formation. The VEGF family is comprised of five distinct ligands, VEGF-A, -B, -C and -D, and the related molecule placental growth factor PlGF. VEGF-A is the dominant member of the family during vascular development. In fact formation of blood vessels has at least two distinct mechanisms during development: firstly *vasculogenesis* where blood vessels are formed *de novo* from endothelial progenitor cells, and *angiogenesis* where vessels form from sprouting of existing vessels. VEGF-A is the critical regulator of each of these processes and therefore is necessary for the development of the organism. Genetic deletion of even a single allele of VEGF in embryonic stem

cells results in embryonic lethality due to vascular malformations (Carmeliet et al., 1996, Ferrara et al., 1996).

In addition to important roles in vascular development VEGF family ligands there is emerging evidence to support a role for continued VEGF signalling in endothelial cells. This signalling has many diverse functions depending on the organ involved and for example endothelial cells have been shown to promote maintenance of the stem cell niche (Hooper et al., 2009, Kobayashi et al., 2010), to control tissue metabolism (Hagberg et al., 2010, Hagberg et al., 2012), and to promote tumorigenesis (Butler et al., 2010). These data indicate that endothelial cells, and VEGF signalling, are more important than simply providing oxygen and nutrients to tissues. Rather these cells are intimately involved in maintaining organ function largely through the provision of paracrine (or angiocrine due to the origin from vascular endothelium) signals.

The structure of VEGFs and related receptors

The VEGFs are a family of homodimeric glycoproteins approximately 40kDa in size. Each of the family members has distinct properties in angiogenesis and in vascular function. As an additional layer of complexity these ligands also display heterogeneity in receptor binding. The patterns of receptor binding to the three main VEGF receptors (VEGFR-1, -2, and -3) are illustrated in **Fig. 1-5**.

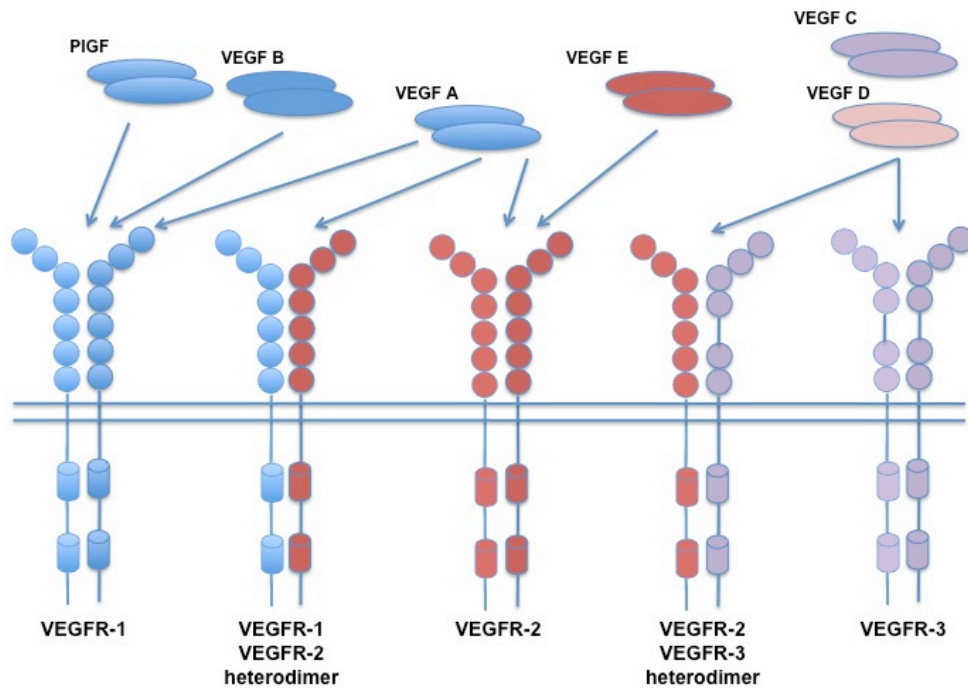


Figure 1-5. Vascular endothelial growth factor (VEGF) receptor specificity.

VEGFs may bind to three receptor tyrosine kinases denoted VEGF receptor (VEGFR) - 1, VEGFR-2 and VEGFR-3 leading to the formation of receptor homodimers and heterodimers. PIGF and VEGF-B bind exclusively to VEGFR1 homodimers whilst VEGF-E binds only to VEGFR-2 homodimers.

VEGF-A is recognised as the major angiogenic growth factor (Nagy et al., 2007), and is the most abundant expressed (Carmeliet and Jain, 2011). VEGF-A is unique in that it binds to both VEGFR-1, and VEGFR-2 whereas VEGF-B, and PlGF bind to VEGFR-1. These ligands also bind to the co-receptor neuropilin-1 that was originally described as an axon guidance molecule (Gu et al., 2003), and that regulates VEGF receptor activation (Fuh et al., 2000, Pan et al., 2007). VEGF-C and -D are primarily involved in lymphangiogenesis and bind to VEGFR-3 (Tammela and Alitalo, 2010). In addition the family members listed above there are additional structurally related molecules that are useful for *in vitro* studies since they bind exclusively to one of the VEGF receptors. One such molecule is VEGF-E that was identified in parapoxvirus and that binds exclusively to VEGFR-2 (Meyer et al., 1999).

The VEGFRs are members of the receptor tyrosine kinase family. For signalling VEGFRs dimerise depending on the ligand bound and are thus able to form both homo- and heterodimers (Dixelius et al., 2003) (**Fig. 1-5**). Following dimerization there is activation of the receptor-kinase activity and recruitment of interacting partners for downstream signalling (Olsson et al., 2006). Importantly the signal transduction mechanisms of different homo-and heterodimers are not completely understood (Waltenberger et al., 1994).

VEGFR-2, also termed fetal liver kinase 1 (FLK-1), is the major receptor for the activation of angiogenesis (Quinn et al., 1993, Shalaby et al., 1995). This receptor is

potently activated by VEGF-A, and, following ligand binding receptor dimerisation and autophosphorylation occur. Downstream signalling leads to a number of outcomes. These include survival and proliferation regulated by tyrosine phosphorylation at position 1175 in human cells and activation of phospholipase-Cy (PLCy) signalling (Takahashi and Shibuya, 1997, Koch et al., 2011). In contrast, permeability and migration is regulated by several different pathways including tyrosine phosphorylation at position 1214 and activation of p38 mitogen-activated protein kinase (MAPK) (Rousseau et al., 1997, McMullen et al., 2005, Koch et al., 2011).

VEGFR-1, also termed FMS-like tyrosine kinase 1 (FLT-1), is a more complex receptor. VEGFR-1 has a much greater affinity for VEGF-A than VEGFR-2 with dissociation constant values (K_d) of 10-20 pM and 75-125 pM respectively (Sato et al., 2000). Perhaps not surprisingly genetic deletion of VEGFR-1 is also embryonically lethal, however in these embryos there is disorganised (and excessive) rather than absent vessel formation (Fong et al., 1995). This finding suggested that VEGFR-1 was involved in vessel patterning and regulation rather than in endothelial cell proliferation *per se*. Indeed *in vitro* studies had previously shown markedly different signal transduction pathways between VEGFR-1 and R-2 (Seetharam et al., 1995), and that phosphorylation of VEGFR-1 was much weaker than that of VEGFR-2 (Waltenberger et al., 1994). To confirm a role in control of angiogenesis Hiratsuka and colleagues deleted the tyrosine kinase domain of VEGFR-1 and expressed the

truncated form in VEGFR-1 null mice (Hiratsuka et al., 1998). These mice developed normally suggesting that the kinase domain is not necessary for VEGFR-1 function and that this receptor rather acts as a decoy for VEGF-A thus preventing aberrant activation of VEGFR-2. Interestingly VEGFR-1 is also alternatively processed to a soluble form that is secreted and may act as an extracellular trap for VEGF-A (Fischer et al., 2008). The functions of soluble VEGFR-1 are not completely understood but expression is increased in a number of diseases including pre-eclampsia – a condition characterised by endothelial cell dysfunction (Levine et al., 2004). VEGFR-1 has also been identified to have a role in pathological angiogenesis, likely due increased expression of PlGF and to cross-talk with VEGFR-2 (Carmeliet et al., 2001, Autiero et al., 2003) although this too is incompletely understood.

1.7 Targeting VEGF in the treatment of human disease

The identification of VEGF-A and its receptors as the critical regulators of angiogenesis lead many researchers to investigate strategies to target this axis with the aim of slowing or preventing the progression of malignant disease. Two main approaches were used to develop these strategies. First neutralising antibodies targeting VEGF-A were developed. The lead antibody bevacizumab (Avastin) was developed by Genentech and was first tested in patients with advanced colorectal cancer in combination with standard of care chemotherapy. This high-profile trial confirmed the benefits of anti-angiogenic therapy but overall the findings were disappointing (Hurwitz et al., 2004). Only a minority of patients responded, and the median improvement in survival was less than five months. The second approach has been to use small molecule inhibitor of receptor tyrosine kinases, including the VEGFRs. This approach has been studied in a number of malignancies but most relevantly in primary liver cell cancer (hepatocellular carcinoma, HCC). In these clinical studies there were again small clinical improvements in the length of overall survival (Cheng et al., 2009, Llovet et al., 2008). Overall these studies have shown that targeting VEGF signalling has some effect, usually by prolonging survival for a few months although those patients who do initially respond later progress.

The mechanisms defining progression of disease despite anti-angiogenic therapy are unclear and several have been suggested (Bergers and Hanahan, 2008). There is

evidence to suggest that endothelial cells can become resistant to anti-VEGF treatment by upregulation of additional angiogenic molecules including FGFs (Casanovas et al., 2005). It has also been suggested that release of angiogenic factors from the tumour stroma can drive blood vessel development in the absence of VEGF-A (Gaengel et al., 2009). Importantly two recent reports suggest that inhibition of VEGF-A signalling before dissemination of cancer cells can accelerate the development of metastasis and the progression of disease (Paez-Ribes et al., 2009, Ebos et al., 2009). This latter finding suggests that VEGF-A signalling may be important in the metastatic niche to prevent the development of metastasis, and when signalling is lost metastasis is permitted more readily.

Finally anti-VEGF therapy is associated with a number of well characterised toxicities including hypertension, bleeding, renal dysfunction, and bowel perforation (Eremina et al., 2008, Hapani et al., 2009, Carmeliet and Jain, 2011, Potente et al., 2011). Some of these toxicities are predictable when one considers the diverse roles of VEGF-A signalling in the endothelium (Lee et al., 2007). In contrast however the risk of gut perforation is not understood and hence there is a need for a better understanding of the role of VEGF-A in the homeostasis of normal tissues and organs.

1.8 The role of VEGF in the response of LSEC to liver injury

VEGF-A expression in the liver is critical for development and for the normal function of LSEC. The potential role for VEGF-A in liver injury was first investigated in partial hepatectomy models of liver regeneration. In these studies expression of VEGF-A was noted soon after resection peaking at approximately 72 hours (Mochida et al., 1998, Sato et al., 2001). At this time after injury there is evidence of LSEC proliferation that is likely mediated by VEGF-A (Shimizu et al., 2001, Taniguchi et al., 2001). The proliferation of hepatocytes occurs earlier, peaking between 24 and 72 hours (Ding et al., 2010), suggesting that this initial proliferation is required to stimulate later LSEC expansion.

Whilst increased VEGF-A expression in hepatocytes is expected to cause LSEC to proliferate in response to injury there are several reports that LSEC may also respond to VEGF-A activation by secreting hepatotrophic factors. In 2003 LeCouter and colleagues reported support of hepatocyte proliferation by endothelial expressed factors, namely interleukin-6 (IL-6), and hepatocyte growth factor (HGF) (LeCouter et al., 2003). Interestingly this was reported to occur by stimulation of LSEC VEGFR-1. The authors were also able to show that VEGF-A primed LSEC were able to ameliorate chemical liver injury suggesting that this mechanism may be active in many forms of liver injury. More recently however these findings have been questioned. Using endothelial cell specific conditional VEGFR-2 knock-out

animals Ding and co-workers identified a role for endothelial cell expressed Wnt2b and HGF in response to VEGF-A stimulation in the regeneration of the liver after partial hepatectomy (Ding et al., 2010). In this model there was no evidence of an effect of VEGFR-1 stimulation. These findings together illustrate the importance of LSEC in liver regeneration and suggest a wider role in the protection of the liver from injury. Specifically the findings further support a role for endothelial cell expressed factors regulated by VEGFR-2 stimulation in the maintenance of normal liver function.

1.9 Epidemiology and natural history of hepatitis C virus infection

Hepatitis C virus (HCV) infection poses a global health problem: as many as 170 million individuals are chronically infected with HCV (Shepard et al., 2005). Many of these individuals will develop progressive disease culminating in the development of cirrhosis, end-stage liver disease, and/or primary liver cancer.

The identification of HCV was predated by a realisation that blood products were contaminated with an agent that caused hepatitis. At this time, hepatitis A, and hepatitis B had been identified and the clinical syndrome was named post-transfusion non-A, non-B hepatitis (Alter et al., 1975, Feinstone et al., 1975). Later HCV was the first virus identified from its genetic material that was identified in the serum of an affected patient (Choo et al., 1989). Since that time the molecular characteristics of HCV have been extensively studied. HCV has been classified as the only member of the *Hepacivirus* genus in the family *Flaviviridae*. There are 6 recognised genotypes that are evolutionarily distinct and that have important clinical differences (Simmonds et al., 2005). The phylogenetic tree of these genotypes is illustrated in **Fig. 1-6**.

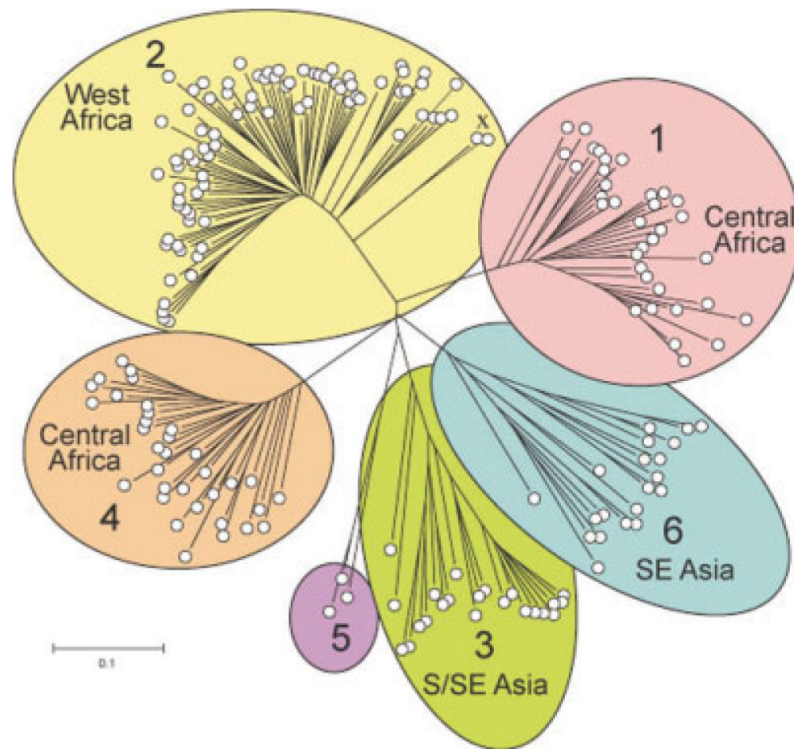


Figure 1-6. Phylogenetic analysis of HCV non-structural protein 5B.

This evolutionary tree illustrates the sequence diversity in the HCV RNA dependent RNA polymerase and the highlights the areas of high-diversity, e.g. Southeast Asia (Simmonds et al., 2005).

In the United Kingdom approximately 230,000 individuals are chronically infected with HCV, largely as a consequence of injecting drug use (Harris et al., 2012). Of those individuals ever infected with HCV approximately 1 in 5 will spontaneously clear infection, a process associated with a vigorous immune response to acute infection (Rehermann, 2009). Of the remaining 80% there is progressive liver injury over the course of many years. At 30 years after infection approximately 30% of individuals will have cirrhosis and be at risk of complications of liver disease (Ferenci et al., 2007). Due to the of patterns of injecting drug use many of these were infected at a young age and there is a large, and growing, group of individuals who have advanced HCV infection and are now beginning to present with complications of liver disease and primary liver cancer (Davis et al., 2010). Since at this stage of disease no medical treatment is available HCV infection is a leading reason to consider liver transplantation (Brown, 2005).

At the early stages of disease individuals chronically infected with HCV often have no symptoms. Thus, only a minority of patients have ever been diagnosed with infection (Culver et al., 2000) and only this subgroup of patients can be considered for treatment. For this reason birth cohort screening has recently been proposed as a method to reduced morbidity and mortality from HCV infection in the United States (Rein et al., 2012). Following identification of infected individuals it is logical to consider antiviral treatment. Indeed the non-specific antiviral interferon alpha was used to treat non-A, non-B hepatitis before HCV was identified (Jacyna et al.,

1989). Over the course of the next 20 years there were pharmacological improvements to that treatment, and the addition of ribavirin but treatment outcomes remained sub-optimal with cure only possible in less than 50% of all patients treated (McHutchison et al., 2009). However recent developments of directly acting antivirals for HCV infection promise much for the outlook of patients with HCV infection. Two medicines, boceprevir and telaprevir, are now licensed for treatment in combination with interferon and ribavirin (Rowe and Mutimer, 2011). These drugs that act to inhibit the viral protease increase cure rates to approximately 65% in genotype 1 but at the expense of an increased side effect profile. There are many other medicines in development that promise to increase cure rates to greater than 80% (Poordad and Dieterich, 2012).

Liver transplantation has proved to be a successful (albeit limited) treatment for patients with end-stage liver disease and HCV infection. Following implantation of the donor organ hepatitis C virions in the plasma rapidly enter the liver to re-establish infection (Garcia-Retortillo et al., 2002, Powers et al., 2006, Dragun et al., 2011). There is always a marked fall in plasma HCV RNA at the time of implantation. One month later levels of plasma HCV RNA have usually increased to greater than 1 \log_{10} higher than pre-transplant. Short-term outcomes after transplant are good but the prognosis of patients with HCV later after transplant is inferior to recipients with other underlying diseases (Mutimer et al., 2006). This is due to progressive fibrosis in the allograft and graft loss due to recurrent disease (Rowe et al., 2008). There is

therefore a need to develop strategies to prevent infection of the allograft at the time of transplantation. In hepatitis B virus infection recurrent disease has been effectively eradicated by a combination of hepatitis B immunoglobulin and specific antivirals. Unfortunately strategies employing HCV immunoglobulin have not been effective (Davis et al., 2005, Schiano et al., 2006) and the focus of attention has now shifted to targeting the host proteins involved in HCV entry into hepatocytes.

1.10 Molecular biology of hepatitis C virus infection

Since the identification of HCV as the causative agent of non-A, non-B hepatitis many investigators struggled to establish cell culture systems to study the HCV lifecycle (Lindenbach and Rice, 2005, Moradpour et al., 2007). The difficulties in visualising the virus, and in identifying infected cells in the liver (either by RNA analysis or immunohistochemistry) initially gave the impression that viral replication was inefficient. However it is now known that plasma titres are usually in the region of 10^6 genome copies per mL, and that approximately 10^{12} viral particles are produced per day (Neumann et al., 1998) indicating that HCV replication is highly efficient *in vivo*.

HCV is a single positive stranded RNA virus of approximately 9.6kb in length. It contains a single open reading frame (ORF) that is translated as a single polyprotein comprising about 3000 amino acids. The ORF is flanked by conserved untranslated regions (UTRs). The 5' UTR contains an internal ribosome entry site (IRES) that initiates translation of the polyprotein (Penin et al., 2004). The polyprotein codes 10 proteins that are cleaved both during, and after translation. These proteins can be separated into the structural, and non-structural proteins (**Fig. 1-8**). Cleavage of the structural proteins is achieved by the endoplasmic reticulum (ER) signal peptidase whilst the non-structural proteins are cleaved by the two viral proteases (Moradpour et al., 2007). The structural proteins that form the physical structure of

the viral particle are the core protein, and the envelope proteins E1, and E2. The non-structural (NS) proteins include p7, an ion channel, the NS2 protease, the NS3 serine protease, NS4B and NS5A, and NS5B, the RNA dependent RNA polymerase. Notably this polymerase lacks a proof reading mechanism and, when coupled with the high levels of replication, this accounts for the high level of genetic diversity that is seen within and between HCV genotypes.

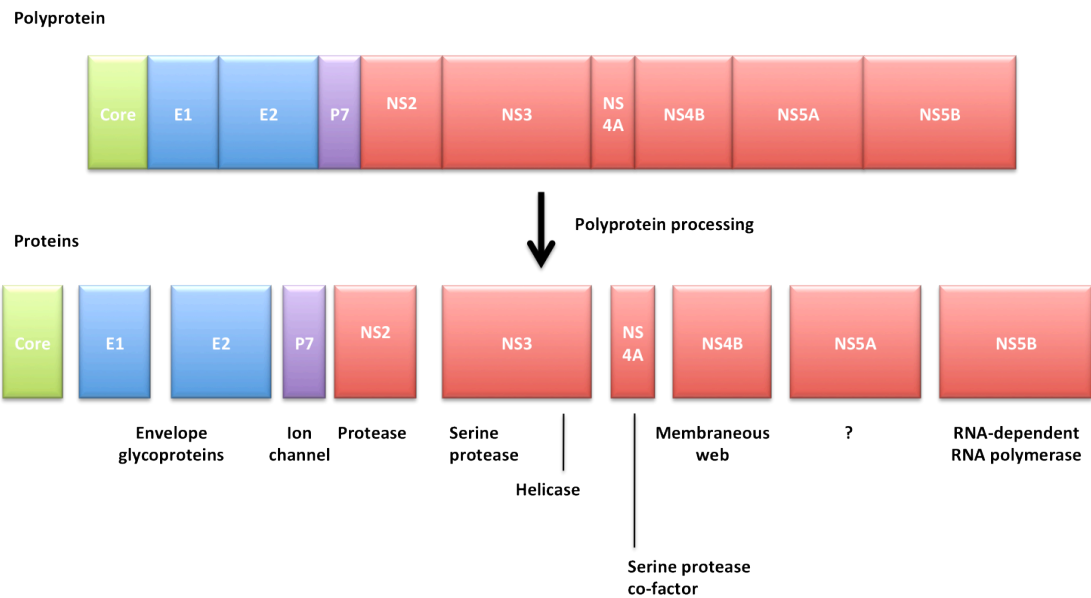


Figure 1-7. Hepatitis C virus polyprotein structure and processing.

The HCV genome encodes a single polyprotein with 3 structural proteins (core, E1 and E2), and 7 non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B). Functions of the individual proteins (where known) are noted below the individual proteins.

Intact HCV particles remain to be well visualised but are approximately 50-65nm in diameter (Shimizu et al., 1996). Virions present in the plasma may be associated with low density lipoprotein (LDL), and very low density lipoprotein (vLDL) and these particles (termed lipoviral particles [LVP]) are likely the infectious fraction (Bassendine et al., 2011). Virions may also be associated with immunoglobulin, or free in the plasma (Thomssen et al., 1993). The association of HCV with vLDL is interesting since it has long been recognised that HCV is frequently associated with lipid accumulation in the liver (Asselah et al., 2006). The relatively recent development of tools to study HCV assembly and release have identified critical roles for pathways related to vLDL synthesis (Gastaminza et al., 2008, Bartenschlager et al., 2011). For example, the role of apolipoprotein E (apoE) in vLDL assembly suggested a role in the HCV lifecycle. Several laboratories have now reported that apoE is required for assembly and virus production (Chang et al., 2007, Benga et al., 2010).

Tools to study hepatitis C virus replication

The first *in vitro* generated tools for studying HCV replication were cDNA clones. The RNA transcripts from these clones were infectious in the chimpanzee – the only animal model thus far identified to be permissive for HCV – after direct inoculation into the liver (Kolykhalov et al., 1997). These clones however were not infectious in cell culture. The most significant step forward in the study of viral replication was in the development of the replicon system (Lohmann et al., 1999). This system initially

used a consensus sequence from an infected human liver but to allow selection of replicating clones the structural region was replaced by a neomycin resistance gene. This development allowed for the first time characterisation of the viral proteins, and the testing of replication inhibitors *in vitro*. Importantly for the efficient replication of HCV a number of mutations (cell culture adaptations) were required (Blight et al., 2000, Lohmann et al., 2001) although the significance of these remains uncertain.

These developments required the use of the human hepatoma cell line Huh-7 for the most efficient replication. Shortly after the replicon system was developed Blight and colleagues established a sub-clone of Huh-7, termed Huh-7.5 that was more permissive for HCV replication (Blight et al., 2002). It was later demonstrated that this was due to a mutation in the retinoic acid-inducible gene I (RIG-I) and a failure of intracellular double stranded RNA sensing in these cells (Sumpter et al., 2005).

To study other aspects of the viral lifecycle, i.e. viral entry, assembly, and release additional tools were necessary. To study viral entry pseudoparticles bearing the HCV envelope proteins were developed and were termed HCVpp. Rather than using the HCV machinery for assembly and replication however, these particles were generated using a retroviral core protein and carry a reporter gene (most frequently luciferase) (Drummer et al., 2003, Hsu et al., 2003, Bartosch et al., 2003a). These

particles enabled the functional studies of the envelope glycoproteins, and also permitted characterisation of the host entry receptors. The initial proof of concept was confirmed by neutralisation of entry by anti-E2 antibodies and serum from patients infected with HCV. These particles, whilst powerful, did not allow study of the complete viral lifecycle.

The ability to study the entire viral lifecycle was realised in 2005 when several laboratories reported a strain of HCV that were able to replicate in cell culture (HCVcc) (Lindenbach et al., 2005, Wakita et al., 2005, Zhong et al., 2005). This genotype 2a strain from a Japanese patient with fulminant hepatitis (termed JFH-1) is able to replicate at high efficiency *in vitro* although the reasons why have not been identified. Importantly the replication of HCVcc in Huh-7.5 cells resulted in the production of infectious viruses into the culture media that could be used to infect both naïve cells, and the chimpanzee (Lindenbach et al., 2006). Later multiple chimaeric viruses were developed expressing the structural proteins of viruses from all of the major genotypes (Gottwein et al., 2009) but replication required the presence of the JFH-1 strain replication complex. This system has accelerated the study and understanding of processes relating to the steps of viral entry post-binding, as well as packaging and release that until the development of HCVcc was near impossible to investigate (Lai et al., 2010, Mancone et al., 2011).

The viral lifecycle

To gain access to cells to permit replication the virus must first interact with specific cellular receptors on the cell surface. Following attachment the particle is internalised by clathrin-dependent endocytosis (Blanchard et al., 2006) and after-pH dependent fusion of the viral and endosomal membranes the encapsulated genome is released into the cytoplasm (Koutsoudakis et al., 2006, Tscherne et al., 2006). This process allows the template RNA to be translated on the endoplasmic reticulum, and then transcribed by the viral polymerase. The viral replication complex is composed of viral proteins, viral RNA, and abnormal cellular membranes that are recognised as a hallmark of all known positive strand RNA viruses (Moradpour et al., 2007). This membraneous web structure was identified as the site of HCV replication and is induced by the actions of NS4B (Gosert et al., 2003, Moradpour et al., 2004). Nascent particles are then assembled and packaged in the endoplasmic reticulum by co-opting pathways of lipoprotein assembly (Bartenschlager et al., 2011). These complete virions are then released into blood to further propagate infection.

1.11 Hepatitis C virus entry receptors

The process of HCV entry is complex and requires the engagement of multiple host cell receptors for internalisation of the virion. The study of HCV entry was accelerated by the development of the HCVpp system and these early reports identified tetraspanin CD81 as the first member of the HCV receptor complex (Hsu et al., 2003). Further receptors, scavenger receptor BI (SR-BI) (Grove et al., 2007, Kapadia et al., 2007), and tight junction proteins claudin-1 (Evans et al., 2007) and occludin (Ploss et al., 2009) have subsequently been identified as necessary components of host cell machinery for viral entry. Latterly additional factors regulating entry have been identified including epidermal growth factor receptor (EGFR), ephrin receptor A2 (EphA2) (Lupberger et al., 2011), and Niemann-Pick C1-like-1 (NPC1L1) (Sainz et al., 2012).

Tetraspanin CD81 – CD81 is ubiquitously expressed on nucleated cells but the physiological functions of CD81 are only partially defined. For instance, it forms part of the B-cell receptor complex but is dispensable for B-cell function. CD81 was identified as a potential receptor for HCV from its ability to bind soluble E2 glycoprotein (Pileri et al., 1998). Subsequent functional assays have shown that both antibodies to CD81, and soluble CD81, potently inhibit infection by HCVpp (Zhang et al., 2004, McKeating et al., 2004, Lavillette et al., 2005) as well as HCVcc (Lindenbach et al., 2005, Wakita et al., 2005, Zhong et al., 2005). Whilst the CD81-

negative Hep-G2 cell line is non-permissive to HCVpp infection, transduction of functional CD81 into these cells restores susceptibility (Flint et al., 2006). Recently studies have shown that ligation of CD81 by antibody, recombinant E2, and HCVcc can stimulate CD81 endocytosis suggesting a role for HCV in stimulating endocytosis and particle internalisation (Farquhar et al., 2012). These findings also suggested that the increase in internalisation of CD81 in HCV infection is related to the involvement of partner proteins since deletion of the C-terminal of CD81 had no effect on receptor internalisation.

Scavenger receptor BI (SR-BI) – SR-BI was first identified for its ability to bind high density lipoprotein (HDL) in the liver (Acton et al., 1996). In a process similar to the identification of CD81 as a cellular receptor for HCV SR-BI was initially shown to bind soluble E2 glycoprotein (Scarselli et al., 2002). Functional studies have confirmed that SR-BI mediates HCV entry in both HCVpp and HCVcc systems (Lavillette et al., 2005, Kapadia et al., 2007, Grove et al., 2007, Dreux et al., 2009). Importantly only SR-BI and not CD81 is able to interact with HCVcc when these proteins are expressed in Chinese hamster ovary cells indicating that SR-BI might act as the primary cellular receptor for HCV infection (Evans et al., 2007). The role of SR-BI in HCV entry has been further confirmed in chimeric mouse model using a well characterised panel of monoclonal antibodies (Catanese et al., 2010, Meuleman et al., 2012, Lacek et al., 2012). These studies have highlighted the therapeutic potential of targeting SR-BI, particularly at the time of liver transplantation. Indeed ITX5061, a chemical inhibitor

of SR-BI (Syder et al., 2011), is under study in patients undergoing transplantation (clinicaltrials.gov NCT01292824).

Claudin-1 (CLDN-1) – Following the development of the HCVpp system it was noted that expression of both CD81 and SR-BI alone was insufficient to render cells permissive to infection indicating that other receptors were required (Bartosch et al., 2003b, Hsu et al., 2003). The tight junction protein CLDN-1 was subsequently identified as a late-step cellular receptor for HCV entry (Evans et al., 2007). Expression of CLDN-1 in cells expressing CD81 and SR-B1 rendered those cells permissive to HCV entry and conversely knockdown of CLDN-1 in cells normally susceptible to HCV entry blocked virus entry. CLDN-1 functions as a tight junction protein and notably is expressed at high levels around the bile canaliculi of hepatocytes (Reynolds et al., 2008). The role of interactions between CD81 and CLDN-1 has been investigated to begin to understand the nature of HCV receptor complexes. These studies have identified direct interactions between these two molecules that are essential for HCV entry (Harris et al., 2010). Interestingly site directed mutagenesis studies have identified amino acid residues on CD81 that are necessary for interactions with CLDN-1 but not for E2 binding confirming a role for protein protein interactions in HCV entry (Davis et al., 2012). Indeed CD81 and claudin-1 have been observed to co-internalise on live-cell imaging (Farquhar et al., 2012). Claudin-1 monoclonal antibodies have also been described (Fofana et al., 2010). These neutralise HCVpp and HCVcc and a role the prevention of graft

infection has been suggested however the critical role of claudins in gut tight junction integrity (Tamura et al., 2011, Wada et al., 2012) provides a cautionary note in the clinical development of these antibodies.

Occludin (OCLN) – The most recently identified cellular receptor for HCV is also a tight junction protein and like CLDN-1 is involved in maintenance of bile canicular structures. OCLN was identified as a receptor for HCV through several strategies including RNA interference of occludin (rendering permissive cells non-permissive) and transduction of normally non-permissive cells with occludin (rendering those cells susceptible to HCV entry) (Ploss et al., 2009, Liu et al., 2009, Benedicto et al., 2008). Direct interactions between E2 and occludin have not been confirmed (Wilson et al., 2012) suggesting that OCLN may form part of a receptor complex rather than directly interacting with the virus to stimulate entry.

Epidermal growth factor receptor (EGFR) and ephrin receptor A2 (EphA2) – These two receptor kinases were identified in a siRNA screen of host cell kinases (Lupberger et al., 2011). The effect of blockade of receptor function was to block the formation of the CD81-CLDN-1 receptor complex and thereby to block the entry of HCV. This was especially relevant since an EGFR kinase inhibitor (erlotinib) is licensed for the treatment of malignant disease. Furthermore the authors showed a modest decrease in HCV RNA load in a chimaeric mouse model treated with erlotinib suggesting a role in the treatment of HCV infection.

Niemann-Pick C1-like-1 (NPC1L1) – This is a cholesterol absorption receptor that has also recently been implicated in HCV entry (Sainz et al., 2012). The function of this receptor *in vivo* is in cholesterol homeostasis and thus in HCV infection NPC1L1 may interact directly with LVPs to remove lipid and promote interaction of E2 with the cellular receptors for HCV, or rather may alter cholesterol levels and membrane function.

There are a number of other receptors that function as possible attachment factors and are present on cell types in the liver. The functions of these molecules are less clear in the process of HCV entry.

Glycosaminoglycans (GAGs) – These polysaccharides present on the surface of many cells that have been shown to bind HCV amongst other viruses (Barth et al., 2006, Germe et al., 2002). The ability to bind HCV is thought to be through interaction with the E2 glycoprotein and occurs preferentially with highly sulphated GAGs such as heparin sulphate (Barth et al., 2003, Barth et al., 2006, Jiang et al., 2012). These molecules are unlikely to be specific receptors for HCV but may facilitate interactions between HCV and its high affinity receptors.

LDL receptor (LDLR) – In the plasma of infected patients, HCV particles are associated with LDL and several investigators have suggested that LDLr is involved in

the uptake of HCV into hepatocytes (Germi et al., 2002, Agnello et al., 1999, Molina et al., 2007). There is some evidence that liver sinusoidal endothelial cells may modulate uptake of HCV-like particles into hepatocytes via the LDLR (Nahmias et al., 2006).

C-type lectins (DC-SIGN and L-SIGN) – These molecules are not expressed on hepatocytes but rather predominantly on dendritic cells or liver sinusoidal endothelial cells respectively (Soilleux et al., 2002, Jameson et al., 2002, Lai et al., 2006). They have been shown to bind HCV and other viruses but due to their pattern of expression they are unlikely to be involved in infection (Lozach et al., 2003, Pohlmann et al., 2003). However, some investigators have reported transmission of DC-SIGN or L-SIGN bound HCVpp to susceptible cells in co-culture and thus these molecules may have a role in capturing HCV and transferring infection to permissive hepatocytes (Lozach et al., 2004, Cormier et al., 2004).

1.12 The role of LSEC in HCV pathogenesis

The liver is the major site of HCV replication. Studies of HCV kinetics on treatment and during liver transplantation indicate that >95% of viral replication occurs in that organ (Powers et al., 2006). In the liver it is the hepatocyte that has been identified as the target cell for HCV however only a minority of hepatocytes (10-20%) appear to be infected at any one time (Powers et al., 2006, Liang et al., 2009). Viral replication is, as noted above, efficient with sufficient particles produced each day (10^{12}) to infect every hepatocyte in the liver. Hepatocytes are typically long-lived and although there is evidence for increased hepatocyte turnover in chronic HCV infection (Marshall et al., 2005) it is highly unlikely that infection rates are that high. Furthermore the half-life of viral particles in the plasma is short (Garcia-Retortillo et al., 2002, Powers et al., 2006) and these divergent aspects of chronic infection together suggest that efficient clearance systems are in place.

The function of LSEC in HCV infection has been largely suggested to involve capture of circulating virions, concentrating these in the sinusoid and permitting access to underlying hepatocytes (Lai et al., 2006, Protzer et al., 2012). Indeed LSEC have previously been shown to bind soluble E2 glycoprotein (Lai et al., 2006) but to date there have been no studies with viruses capable of the complete lifecycle *in vitro*. The roles of LSEC as a dynamic sieve, and as a clearance mechanism suggests rather that LSEC will permit access to hepatocytes through fenestrated sieve plates, and

will clear particles that are taken up from the circulation (Elvevold et al., 2008, Ganesan et al., 2011). The fate of these particles is not clear but the mathematical modelling indicates that large scale clearance is occurring, and the biology of LSEC suggests that it is these cells that are responsible.

1.13 Aims of the project

The overarching aim of this project was to understand the role of liver sinusoidal endothelial cells (LSEC) in hepatitis C virus infection (HCV) of the liver. The specific objectives of the studies were to firstly understand the role of LSEC in the initial infection of hepatocytes, and secondly to define the role of LSEC in chronic infection through investigation of paracrine signalling systems *in vitro*.

Chapter 2 Materials and methods

2.1 Tissue samples

All tissue samples were from the Queen Elizabeth Hospital Birmingham Liver and Hepatobiliary Unit. All material was collected in accordance with local research ethics approvals and with the consent of patients or their relatives. Diseased liver was from patients undergoing liver transplantation for various indications whilst normal tissue was from tissue surplus to requirements at transplant, or from macroscopically normal liver removed during resection of secondary liver tumours. Tissue was processed fresh for isolation of constituent cell types: liver sinusoidal endothelial cells, biliary epithelial cells, or activated liver myofibroblasts. For messenger RNA and protein analysis 1x1x1cm cubes of liver were snap frozen over liquid nitrogen and stored at -80°C until required for processing.

2.2 Isolation of liver sinusoidal endothelial cells

An enzymatic method was used to isolate liver sinusoidal endothelial cells (LSEC) (Lalor et al., 2006, Liaskou et al., 2010, Shetty et al., 2011). Approximately 30g of liver tissue was cut into small pieces and digested with a type-1A collagenase digestion (2mg/mL, Sigma Aldrich Ltd., Poole, UK) for 45 minutes at 37°C. Undigested tissue was removed by passing the digestate through a sterile fine mesh. The remaining suspension was sedimented by centrifugation. This crude cell preparation was then separated over a Percoll gradient (Amersham Biosciences, GE) at 450g for 25 minutes. Biliary epithelial cells were removed by indirect magnetic separation using an antibody raised against the epithelial cell surface specific glycoprotein HEA-125 (10µg/mL, Progen Biotechnik,) followed by a secondary antibody conjugated to magnetic beads (sheep anti-mouse Dynabeads, Dynal, Invitrogen). Sinusoidal endothelial cells were isolated from the remaining suspension by a second immunomagnetic separation using antibody raised against CD31 (10µg/mL) directly conjugated to magnetic beads.

During this process those biliary epithelial cells isolated were retained. The remaining cell suspension following isolation of biliary epithelial cells and LSEC contained activated liver myofibroblasts and was also retained for studies of gene expression.

2.3 Cell culture

All tissue culture work was done in class II microflow safety cabinets. Aseptic techniques were used at all times. All cells were maintained at 37°C and 5% CO₂.

Primary cells

Isolated LSEC were cultured in endothelial basal media (Invitrogen, UK) supplemented with 10% heat inactivated normal human AB serum (HD Supplies, Bucks, UK), 10ng/mL hepatocyte growth factor (HGF), 10ng/mL vascular endothelial growth factor A (VEGF-A, both PeproTech, UK), and 50units/mL benzylpenicillin streptomycin (Gibco) in type I collagen (Sigma-Aldrich) coated flasks.

Activated liver myofibroblasts (aLMF) were cultured in gelatin-coated flasks in Dulbecco's modified Eagle medium (DMEM) (Gibco, California, USA), supplemented with 10% foetal bovine serum, 1% glutamine, and 50units/mL benzylpenicillin/streptomycin.

Biliary epithelial cells (BEC) were cultivated on type I collagen coated flasks. Growth media consisted of DMEM supplemented with 10% human serum, and 50units/mL benzylpenicillin/streptomycin. In addition, the following growth factors and drugs

were added: epidermal growth factor (Peprotech, 10ng/mL), HGF (10ng/ml), triiodothyronine (Sigma-Aldrich, 2nM), insulin (Sigma-Aldrich, 0.124U/mL), hydrocortisone (Queen Elizabeth Hospital Birmingham, 2µg/mL), and cholera toxin (Sigma-Aldrich, 10ng/mL). For each of these non-parenchymal cell types spent media was replaced every 3 days.

Primary human hepatocytes were a gift from Dr Ragai Mitry (King's College Hospital, London). These were isolated according to published protocols (Mitry, 2009, Mitry et al., 2010) and maintained in Williams Essential Eagles Medium (Sigma-Aldrich), supplemented with 10% human serum, 1% L-glutamine, 1% non-essential amino acids and 50units/ml benzylpenicillin/streptomycin. Spent media were replaced daily.

Human umbilical vein endothelial cells (HUVEC) were a gift from Professor Roy Bicknell. These cells were isolated according to laboratory protocols, and were maintained in human endothelial basal media, supplemented with 10% heat inactivated normal human AB serum and 50units/ml benzylpenicillin/streptomycin on gelatin coated tissue culture plastic for the purposes of experiments outlined in this thesis.

Cell lines

Huh-7.5 cells (provided by C. Rice, Rockefeller University, New York, USA) were cultured in DMEM, supplemented with 10% foetal bovine serum, 1% L-glutamine, 1% non-essential amino acids and 50units/mL benzylpenicillin/streptomycin.

Chinese Hamster Ovary (CHO) cells were from the American Type Culture Collection, Virginia USA and were maintained in F12 medium (Invitrogen) supplemented with 1% non-essential amino acids and 50units/ml benzylpenicillin/streptomycin (Gibco). CHO cells stably expressing SR-BI were previously generated as described (Grove et al., 2007).

Cell culture

Cells were cultured in conditions described above and growth was assessed using an inverted phase contrast microscope. The cells were monitored daily for normal cell morphology, changes in media colour and clarity, and cell density. When appropriate cells were passaged using proteolytic enzymes: TrypLE (Trypsin like enzyme Express, Invitrogen) for primary cells, and trypsin (Gibco) for cell lines. Culture media was discarded and cells washed in sterile phosphate buffered saline (PBS). An appropriate volume of proteolytic enzyme was added and cells were incubated for 1-5 minutes before gentle agitation to ensure that cells were detached. PBS was then added to the cells and the suspension centrifuged at 550g for 5

minutes. The pelleted cells were then resuspended in fresh growth media into (where necessary, coated) tissue culture plastic flasks.

Freezing cells

Stored cells were preserved in liquid nitrogen. After pelleting the cells as above cells were resuspended in freezing media (95% FBS, 5% DMSO [Sigma-Aldrich]) and transferred into cryovials for freezing. Cryovials were placed in a MrFrosty freezing container (Wessington Cryogenics) and transferred to a -80°C freezer. After overnight storage the cryovials were transferred to liquid nitrogen.

When cells were required for experiments cryovials were removed from liquid nitrogen, thawed, washed in PBS, and centrifuged to remove cellular debris. Cells were then counted and viability assessed using trypan blue exclusion. Cells were resuspended in appropriate culture media and plated on (coated) tissue culture plastic.

2.4 Antibodies and reagents

The antibodies used in these studies are listed in **Table 2-1**, and growth factors and proteins are listed in **Table 2-2**.

Primary antibodies			
Antigen	Clone	Application(s)	Source
CD81	2s.131	FC	In house
SR-BI	R25	FC	T.Huby, Université Pierre et Marie Curie
Claudin-1	Polyclonal sera	FC	T Baumert, Université de Strasbourg
Occludin	OC-3F10	FC	Invitrogen
L-SIGN	604	IF	R&D Systems
NS5A	9E10	IF	C Rice, Rockefeller University
VEGFR-1	IMC-18F1	Neutralisation	Imclone
VEGFR-1	321	WB	R&D Systems
VEGFR-2	IMC-1121	Neutralisation	Imclone
VEGFR-2	55B11	WB/IHC	Cell Signalling
Phospho-VEGFR-2 (Tyr1175)	19A10	WB	Cell Signalling
BMP4	757	WB/Neutralisation	R&D Systems
β -actin	AC-74	WB	Sigma Aldrich Ltd.
VEGF-A	293	Neutralisation	R&D Systems
p38 MAPK	9212	WB	Cell Signalling
Phospho-p38 MAPK	9211	WB	Cell Signalling
Secondary antibodies			
Name	Antigen	Application(s)	Source
Mouse Alexa Fluor 488	Mouse IgG	IF/FC	Molecular Probes, Invitrogen
Rat Alexa Fluor 488	Rat IgG	IF/FC	Molecular Probes, Invitrogen
Mouse/rabbit universal	Mouse/rabbit IgG	IHC	Vector Laboratories
Anti-mouse-HRP	Mouse IgG	WB	GE Healthcare
Anti-rabbit-HRP	Rabbit IgG	WB	GE healthcare
Anti-goat-HRP	Goat IgG	WB	Santa Cruz Biotechnology

Table 2-1. Antibodies used in this study.

Applications listed are: FC, flow cytometry; IF, immunofluorescence; IHC, immunohistochemistry; WB, western blotting; and neutralisation.

Name	Source	Concentration
VEGF-A	Peprotech	Various
VEGF-E	RELIATech	10ng/mL
PlGF	Peprotech	10ng/mL
BMP4	Peprotech	Various
HGF	Peprotech	10ng/mL
PD98059	Sigma-Aldrich	10 μ M
SB203580	Sigma-Aldrich	Various
Wortmannin	Sigma-Aldrich	10 μ M
U73122	Sigma-Aldrich	10 μ M

Table 2-2. Growth factors and chemicals used in this study.

2.5 Routine techniques

Indirect immunofluorescence

Liver sinusoidal endothelial cells were seeded at 4×10^4 cells per well on type I collagen coated borosilicate glass coverslips and cultured until confluent. Cells were fixed in ice-cold methanol for 5 minutes, washed and then permeabilised with PBS containing 0.5% bovine serum albumin and saponin for 30 minutes. Cells were incubated with primary antibody for one hour in PBS, 1% BSA, and 1% saponin. Cells were then washed three times and incubated with the appropriate Alexa-488 conjugated anti-species secondary antibody diluted 1:1000 in PBS-BSA-saponin at room temperature. Finally cells were washed three times in PBS-BSA-saponin, counterstained with 4',6'-diamidino-2-phenylindole (DAPI) (Invitrogen) in PBS for 5 minutes and coverslips mounted on glass slides (ProLong Gold Antifade, Invitrogen). Slides were viewed with a Nikon Eclipse TE2000-S microscope at a magnification of x200 and images taken using a Hamamatsu C4742-65 camera (Nikon).

Flow cytometry for HCV receptor expression

For CD81, SR-BI and claudin-1 staining 2×10^5 cells were incubated in phosphate-buffered saline containing 1% bovine serum albumin and 0.01% sodium azide. For occludin staining 2×10^5 cells were fixed in 1% paraformaldehyde and incubated in phosphate-buffered saline containing 1% BSA and 1% saponin. 2µg receptor specific

antibody or irrelevant IgG controls were incubated in cells in azide for 30 minutes. Unbound antibody was removed by washing. Secondary anti-mouse, anti-rat or anti-rabbit Alex-488 conjugated antibodies were incubated with cells for a further 30 minutes and the cells washed and fixed in 1% paraformaldehyde. Antibody binding was assessed by flow cytometry using a FACSCalibur (BD Biosciences) and data were analysed with FlowJo software (TreeStar).

Western blotting

LSEC were lysed *in situ* in ice-cold lysis buffer (CellLytic MT, Sigma-Aldrich) containing protease (Complete, Roche), and phosphatase (Roche) inhibitors for 30 minutes on ice. Whole liver samples were homogenised in M-tubes (Miltenyi) using a gentleMACS dissociator (Miltenyi). Lysates were clarified by centrifugation (20,000xg for 15 minutes) and the supernatant stored at -20°C. Protein concentration was determined using the Protein Assay Reagent (Pierce) according to the manufacturer's instructions (**Fig. 2-1**).

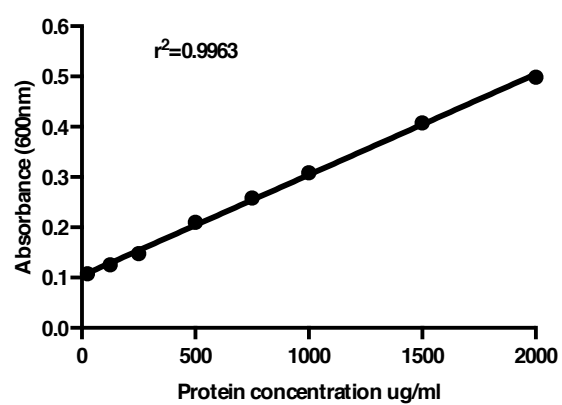


Figure 2-1. BCA protein assay standard curve.

7% sodium dodecyl sulphate-polyacrylamide gel electrophoresis gels were cast and samples of equal protein content were prepared by adding equal amounts of protein to 4x Laemmli loading dye (H₂O, 30% v/v glycerol, 6% SDS, 0.2% v/v bromophenol blue and 0.2M Tris-HCV; pH 6.8) with (reducing conditions), or without (non-reducing conditions) 10% 2-β-mercaptoethanol. The total volume was adjusted to 20μL with H₂O and samples heat denatured at 95°C for 5 minutes. Samples were cooled before loading.

20μg of protein lysates were loaded in addition to a prestained standard (Novex Sharp, Invitrogen). Protein electrophoresis was performed at 200V for 35 minutes using the Mini Protean 3 system (Bio-Rad Laboratories) and transferred to polyvinylidene difluoride membranes (Millipore) at 350A for 60 minutes. Membranes were blocked with 5% skimmed milk, or 5% BSA and subsequently incubated in blocking buffer with primary antibody. The blots were washed and incubated with appropriate secondary antibodies conjugated to horseradish peroxidase. The membranes were then repeatedly washed and immunoreactive bands detected using enhanced chemoluminescence (Pico SuperSignal, Pierce).

Cell proliferation assay

LSEC were seeded at 2×10^4 cells per well in a type I collagen coated 24-well plate in endothelial basal media containing 10% heat inactivated normal human serum,

60µg/mL benzylpenicillin and 100µg/mL streptomycin. After 16 hours LSEC were treated in duplicate for 72 hours with recombinant VEGF-A, VEGF-E, or PlGF. Following treatment LSEC were washed and incubated in 200µl endothelial basal media containing 10% heat inactivated normal human AB serum, 60µg/ml benzylpenicillin and 100µg/ml streptomycin plus 10µl 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) (Promega). After 2 hours 100µl of the MTS solution was removed and transferred to a 96-well assay plate and absorbance measured at 490nm in a microplate photometer (Multiskan Ascent, Fischer Thermo Scientific). Relative cell number was calculated by subtracting the mean absorbance from MTS solution alone from the readings for the LSEC samples and then normalised to the untreated LSEC sample, i.e. the mean absorbance of the untreated LSEC sample was defined as 1.

Immunohistochemistry

Tissue blocks fixed in formalin were obtained from tissue unused for transplantation. Representative 3µm sections were placed onto charged slides (Surgipath) and stored at room temperature until required. Sections were de-parafinised for 10 minutes in Xylene (Surgipath) and then rehydrated in H₂O. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide in H₂O. Epitopes were retrieved by placing sections in EDTA (pH 8.0) and this was then microwaved for 20 minutes and allowed to cool.

Sections were then mounted onto a Shandon Sequencer (Thermo Scientific). These were blocked in 2% casein (Vector Labs) diluted in Tris-buffered saline (pH 7.5). Specimens were incubated with antibodies specific for VEGFR-2 or an irrelevant isotype control overnight at 4°C. Antibodies were diluted in Tris-buffered saline with 0.1% Tween (TBS-T) and 5% goat serum.

Specimens were washed with TBS-T three times and then incubated with secondary antibody reagent (ImPress Universal anti-mouse IgG/anti-rabbit IgG peroxidase kit, Vector Labs) according to the manufacturer's instructions. Specimens were again washed three times in TBS-T and antibodies were visualised with ImPact DAB Diluent and Chromogen Kit (Vector Labs). Sections were then counterstained with haematoxylin (Surgipath). Slides were mounted after xylene dehydration and were sealed with 24mm coverslips using DPX mounting reagent (VWR). Slides were dried in a laminar fume cupboard overnight. Images were obtained the following day using a Nikon Eclipse E400 microscope (Nikon). Immunohistochemistry studies were performed with the help of Dr Garrick Wilson.

Messenger RNA extraction

Cell preparations (4×10^4 cells) were lysed *in situ* whilst whole liver whole liver tissue was disrupted in an M-tube and using the gentle MACS dissociator. Total cellular

RNA was extracted using the RNeasy mini kit (Qiagen) according to the manufacturers instructions.

Quantitative Reverse Transcriptase Polymerase Chain Reaction

Purified RNA was amplified for various target genes, including *VEGF-A*, *BMP4*, *VEGFR-1*, and *VEGFR-2* with commercially available quantification kits (Applied Biosystems). HCV RNA was amplified using primers targeting a conserved region of core (Primer Design).

Reactions were conducted in triplicate for each condition in a single tube RT-PCR in a MicroAmp 96 optimal reaction plate (Applied Biosystems), and in accordance with the manufacturers instructions (Cells Direct Kit, Invitrogen). Fluorescence was monitored in a Stratagene RT-PCR machine (MX3000P, Stratagene, Agilent). In all reactions the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included as an internal endogenous control for amplification efficiency and RNA quantification (primer-limited endogenous control; Applied Biosystems). The PCR reaction was done using the following program: 30 mins at 50°C, 5 mins at 95°C, followed by 50 cycles of 15 seconds at 95°C and 60 seconds at 60°C.

In experiments to quantify HCV RNA a pre-prepared standard (Primer Design) was included as shown in **Fig. 2-2**.

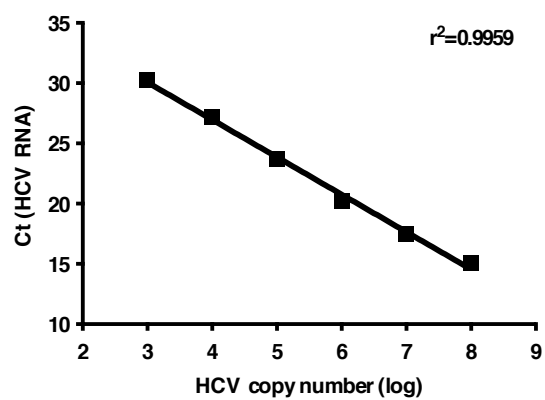


Figure 2-2. HCV RNA standard curve.

2.6 HCVpp generation and infection

Hepatitis C virus pseudoparticles (HCVpp) were generated according to the method of Hsu (Hsu et al., 2003). 293T cells (American Type Culture Collection, Virginia, USA) were transfected with two plasmids, one encoding human immunodeficiency provirus expressing luciferase and the other containing the HCV strain H77 glycoproteins (E1 and E2), the vesicular stomatitis virus (VSV) envelope, or the murine leukaemia virus (MLV) envelope, or a no-envelope control. Virus containing supernatants were harvested after 48 hours, clarified and sterile filtered through a 0.45µm membrane. To assess HCV entry diluted virus containing supernatant was incubated with target cells for 8 hours in triplicate wells, unbound virus removed, and culture maintained in target cell specific medium. At 72 hours post infection media was removed and cells lysed in cell lysis buffer (Promega). Luciferase activity was detected after the addition of luciferase substrate and measurements were taken for 10 seconds in a luminometer (Lumat LB9507). Infectivity expressed as relative light units was calculated by subtracting the mean no-envelope control signal from the HCVpp, VSVpp or MLVpp signals.

2.7 HCVcc generation and infection

Plasmids encoding full-length HCV strain JFH-1 (provided by T. Wakita, National Institute of Infectious Disease, Tokyo, Japan) (Lindenbach et al., 2005, Wakita et al., 2005, Zhong et al., 2005) was used to generate RNA as previously described. RNA transcripts were generated using the T7 RNA polymerase kit (Promega) according to the manufacturers instructions and electroporated into Huh-7.5 cells. To determine productive infection cells were fixed in ice-cold methanol and stained for NS5A with the anti-NS5A monoclonal antibody (9E10 [provided by C. Rice, Rockefeller University]) and an Alexa 488-conjugated anti-mouse IgG (Molecular Probes, Invitrogen, **Fig. 2.3**). The supernatants containing HCVcc were collected at 72 and 96 hours post infection, pooled and stored immediately at -80°C.

Virus containing supernatant was used to infect target cells: cells were incubated with HCVcc for four hours washed and then culture maintained for 48 or 72 hours as specified in the figure legends. Infection was quantified by enumerating NS5A positive foci in triplicate wells and infectivity defined as the number of focus forming units per millilitre of inoculum.

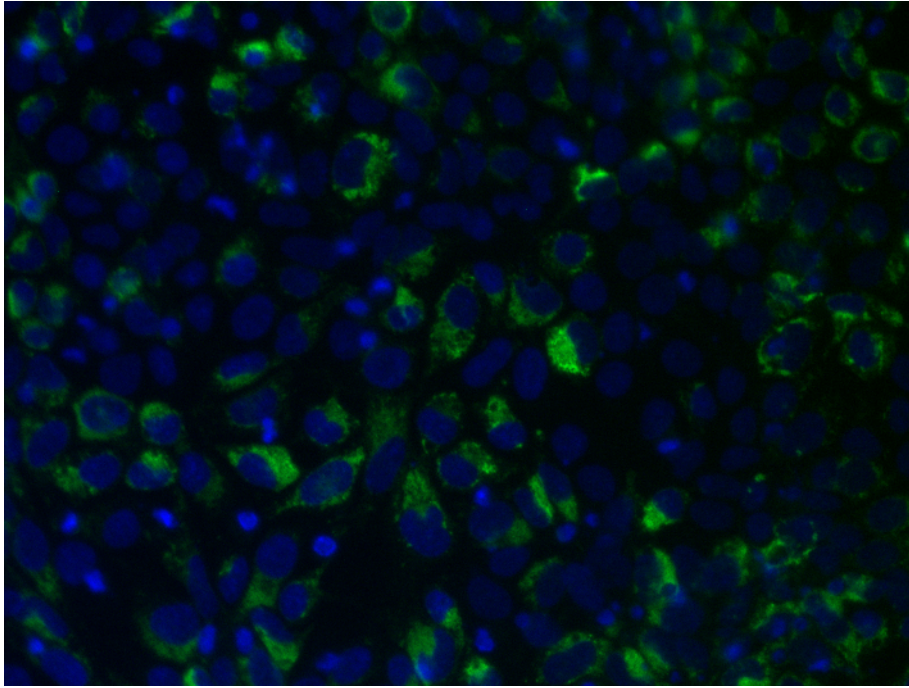


Figure 2-3. Electroporated Huh-7.5 cells stained for viral antigen NS5A.

Huh-7.5 cells were electroporated with JFH-1 RNA and 48 hours later were fixed in ice-cold methanol and stained for NS5A. Original magnification x200.

2.8 Liver sinusoidal endothelial cell transfer of infection to Huh-7.5 cell

Isolated LSEC, CHO cells and Huh-7.5 cells were seeded at 2×10^4 cells per well of a 24-well plate and maintained for 16 hours. The cells were then incubated with HCVcc for two hours at 37°C and then washed extensively to remove any unbound viral particles. 2×10^4 Huh-7.5 cells per well were labelled with $5 \mu\text{M}$ 5-chloromethylfluorescein diacetate (CMFDA) according to the manufacturers instructions (Invitrogen) and then added to the LSEC, CHO cell or Huh-7.5 cell monolayers and culture maintained for a further 72 hours in VEGF-A free LSEC medium. After this time the cells were fixed in ice-cold methanol and stained for NS5A. This is summarised in **Fig. 2-4**.

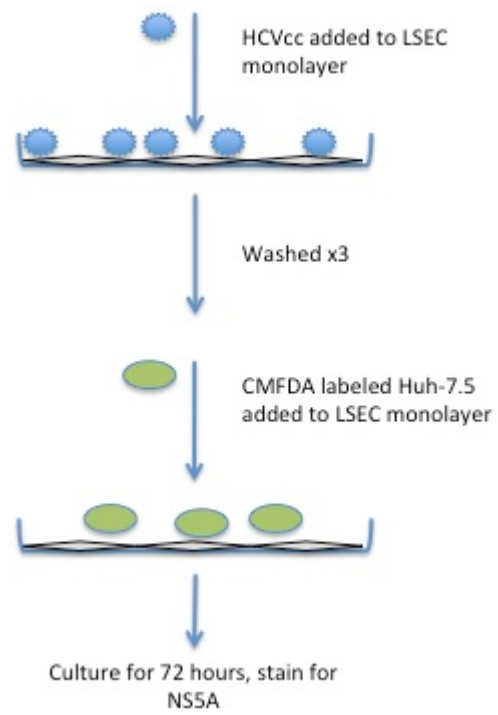


Figure 2-4. Transfer of infection from LSEC to Huh-7.5 cells.

2.9 Conditioned media

Conditioned media was generated by seeding 4×10^4 cells per well of a 24-well plate in 1ml media. For LSEC cultures were maintained in endothelial basal media containing 10% heat inactivated human serum, 50units/ml benzylpenicillin/streptomycin. After 24 hours conditioned media was carefully removed, aliquoted, and stored at -20°C .

For the treatment of Huh-7.5 cells conditioned media was diluted 1:2 with fresh media. Conditioned media were then incubated with Huh-7.5 cells overnight before incubation for 4 hours with HCVcc as described above. Following this conditioned media were again incubated with Huh-7.5 cells for the duration of infection.

Conditioned media were also fractionated using size exclusion membranes in manufactured 15mL tubes (VivaSpin 6, Sartorius). Membranes excluding proteins >100 , >50 , >30 , and >10 kDa were used. The media retrieved from above the membrane were diluted with fresh media to equalise the volume of media to that of the input. These media were further diluted 1:2 with fresh media to treat Huh-7.5 cells as above.

2.10 Co-culture of liver sinusoidal endothelial cells and Huh-7.5 cells

LSEC and CHO cells were seeded at 2×10^4 per cm^2 in a type I collagen coated 48-well plate in endothelial basal media containing 10% heat inactivated normal human AB serum, 60 $\mu\text{g}/\text{ml}$ benzylpenicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. After two hours Huh-7.5 cells were added at 2×10^4 per cm^2 and cultured for 16 hours. Huh-7.5 cell monoculture was established by seeding 4×10^4 per cm^2 in a type I collagen coated 48-well plate in endothelial basal media containing 10% heat inactivated human serum, and 50units/ml benzylpenicillin/streptomycin for 16 hours. After this time cells were incubated with HCVcc JFH-1 for two hours, following this cells were washed and media replaced with endothelial basal media containing 10% heat inactivated human serum, 50units/ml benzylpenicillin/streptomycin for 48 hours. Cells were then fixed in ice-cold methanol and stained for NS5A.

For experiments where co-culture of LSEC and Huh-7.5 cells was required without heterotypic cell contact specially designed slides were used (Ibidi 2x9 well μ -Slide) according to the manufacturers instructions. Briefly, LSEC and Huh-7.5 cells were diluted to 7.5×10^4 per mL and 50 μl of the cell suspension was added to each of the wells as illustrated in **Fig. 2-5**. The LSEC were seeded in the central minor well and the Huh-7.5 cells were seeded in the surrounding wells. After two hours the major well was flooded with 500 μl of media to allow exchange of soluble mediators between cell types.

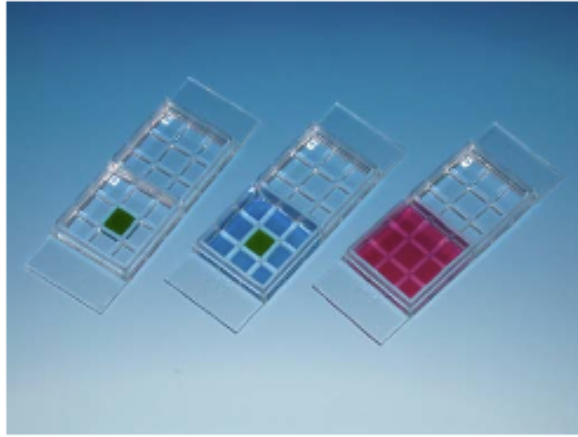


Figure 2-5. Ibidi 2x9 well μ -Slide for co-culture without heterotypic cell contact.

LSEC were seeded in the central well, in this example coloured green. Huh-7.5 cells were then seeded in the surrounding wells, coloured blue. After attachment the major well was flooded with media to allow exchange of soluble factors.

2.11 Endothelial cell microarray

Sample preparation

LSEC and HUVEC, each from 2 independent donors, were grown in duplicate to confluence on gelatin coated 10cm tissue culture plastic plates in their usual growth media. At pre-confluence the media was changed to endothelial basal media supplemented with 10% human serum, and 50units/ml benzylpenicillin/streptomycin. Following culture for 24 hours the supernatant was replaced with untreated, or VEGF-A (10ng/mL) treated endothelial basal media supplemented with 10% human serum, and 60µg/ml benzylpenicillin and 100µg/ml streptomycin. Treatment was maintained for 18 hours and cells were then lysed *in situ* for RNA extraction as described above.

Analysis of RNA quality

RNA extracted from each of the samples was quantified (NanoDrop, Thermo Scientific) and the quality checked using a Bioanalyser machine (2100 Bioanalyser, Agilent). The outputs of these analyses are illustrated in **Fig. 2-5**. The RNA used for microarray analysis was all of high quality. RNA integrity number (RIN) values obtained using Bioanalyser analysis were all >9.5 indicating that there was no significant RNA degradation and initial concentrations of RNA isolated were in the range 400-1000ng/µL.

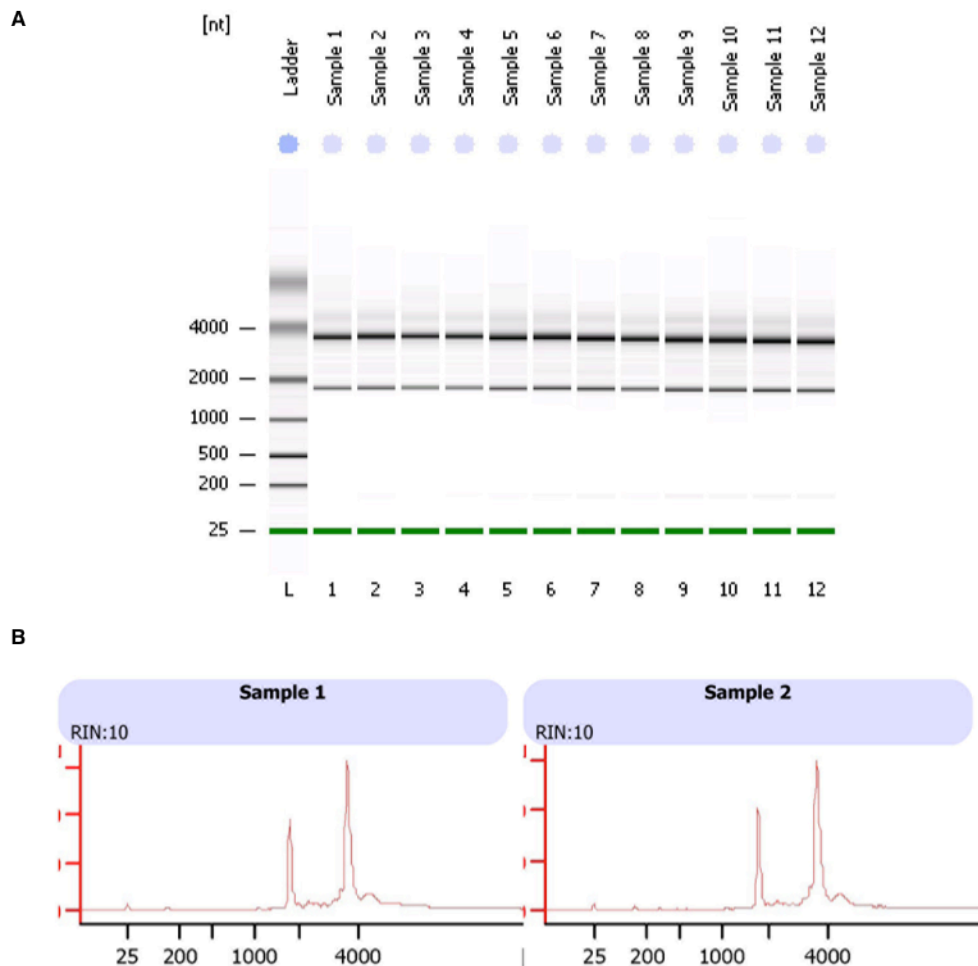


Figure 2-6. Bioanalyser output.

Indicative output of endothelial cell messenger RNA analysed for RNA quality. Electropherogram of samples included in microarray analysis: Sample 1-4 LSEC plus VEGF-A stimulation, Sample 5-8 HUVEC minus VEGF-A stimulation, and Sample 9-12 HUVEC plus VEGF-A stimulation (**A**). Graphical interpretation of the electropherogram for samples 1 and 2 (**B**).

RNA labelling and hybridisation

Total messenger RNA was labelled using the Quick Amp Labelling Kit (Agilent) according to the manufacturer's instructions. Untreated endothelial cell complementary RNA was labelled with Cy3, and VEGF-A treated samples were labelled with Cy5. A diagrammatic summary of the process is provided in **Fig 2-6**. Following labelling complementary RNA was purified using an RNeasy kit.

Hybridisation was done using a Gene Expression Hybridisation Kit (Agilent) in conjunction with human 4x44k oligo arrays (Agilent) and according to the manufacturer's protocol. 825ng of each labelled sample was using in the hybridisation protocol. The hybridisation was performed for 17 hours at 65°C at 10 revolutions per minute in a hybridisation oven. Following hybridisation the microarray slides were washed according to the manufacturer's protocols in wash buffers 1, and 2, and then in wash buffer 2 at 37 °C, each for one minute.

The slides were scanned immediately on an Agilent 2505C scanner and images were quantified using the Agilent Feature Extraction software (v. 10.5.1.1).

Bioinformatic analysis

Extracted data were analysed using GeneSpring GX (v. 7.3.1, Silicon Genetics). The normalised log₁₀ ratio (Cy5/Cy3) representing the target to reference ratio was calculated. Genes regulated more than 1.8-fold, with a false discovery rate of <0.05 were selected for further analysis.

Microarray data were deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus database with the accession number GSE41110. The record is available to view at:

<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=hpgzpgiigemmynm&acc=GSE41110>

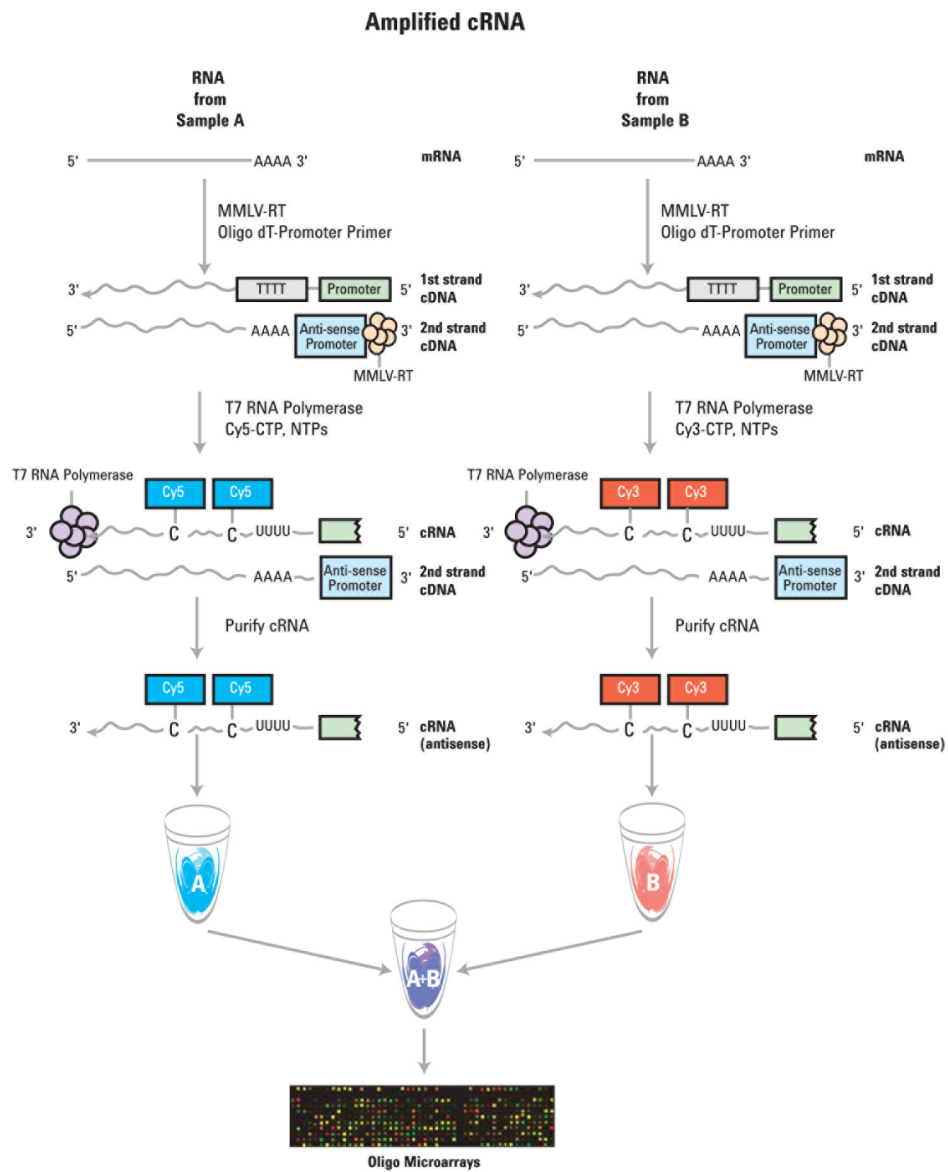


Figure 2-7. Schematic diagram of messenger RNA processing for 2-colour microarray analysis.

2.12 Plasma HCV RNA quantitation during liver transplantation

Patients enrolled in the ITX5061 in liver transplant recipients study gave informed consent to blood sampling during liver transplantation (clinicaltrials.gov NCT01292824). Samples were taken at admission to hospital, at induction of anaesthesia, at the time that there was no blood flow through the liver and the patient was functionally anhepatic (literally no liver), and 30 minutes later. Further samples were taken immediately before blood flowed through the implanted liver (reperfusion), and 1 hour after that. Once the patient returned to the intensive care unit further samples were taken at 4 hourly intervals in the first day after transplantation.

Plasma samples were sent directly to the regional Health Protection Agency Laboratory at Birmingham Heartlands Hospital for analysis. HCV RNA was quantified using the Roche Cobas Amplicor 2.0 assay.

2.13 Statistical analysis

Results are shown as the mean \pm standard deviation unless otherwise stated. Data were compared using non-parametric statistics since normality of small samples cannot be readily established. For comparisons of two groups the Mann-Witney U test was used. For experiments containing multiple comparisons data were compared with the Kruskal-Wallis test using Dunn's correction for multiple comparisons. Linear regression analyses were used as indicated to explore relationships between continuous variables, and to calculate protein and RNA concentrations from standard curves. All statistical analyses were done with Prism 5.0 (GraphPad).

Chapter 3 Liver sinusoidal endothelial cells restrict HCV infection

The liver is a large and complex organ containing multiple cell types including LSEC, biliary epithelial cells, stellate and Kupffer cells, as well as hepatocytes. Hepatocytes are the only cell type in the liver known to support HCV replication and the majority of published studies are limited to this cell type. In the liver lobule hepatocytes are in close proximity to LSEC and blood flowing through the liver is distributed through the sinusoids. Thus HCV particles in the blood are likely to encounter LSEC and it is important to understand the role of LSEC in the virus lifecycle.

LSEC have been reported to express HCV receptor CD81 and the lectin L-SIGN and to bind recombinant viral envelope proteins or virus like particles (Lai et al., 2006, Nahmias et al., 2006). Since these studies indicated that LSEC express receptors capable of binding HCV it has been suggested that the major role of LSEC is to capture circulating virions and potentiate the infection of underlying hepatocytes (Cormier et al., 2004, Lai et al., 2006, Lozach et al., 2004). The development of the HCVcc system permits investigation into the role of non-parenchymal cells in HCV replication and I established co-culture model systems to study the role of LSEC in HCV lifecycle.

3.1 Phenotypic characteristics of isolated liver sinusoidal endothelial cells

LSEC were isolated from whole liver as previously described (Lalor et al., 2006, Liaskou et al., 2010, Shetty et al., 2011). In culture these cells maintained classical endothelial cell morphology until at least passage 4 (**Fig. 3-1A**). Isolated cells expressed calcium dependent the c-type lectin L-SIGN that is reported to be highly expressed on liver endothelial cells *in vitro* and has been advocated as an excellent marker of this cell type (Lalor et al., 2006) (**Fig. 3-1B**).

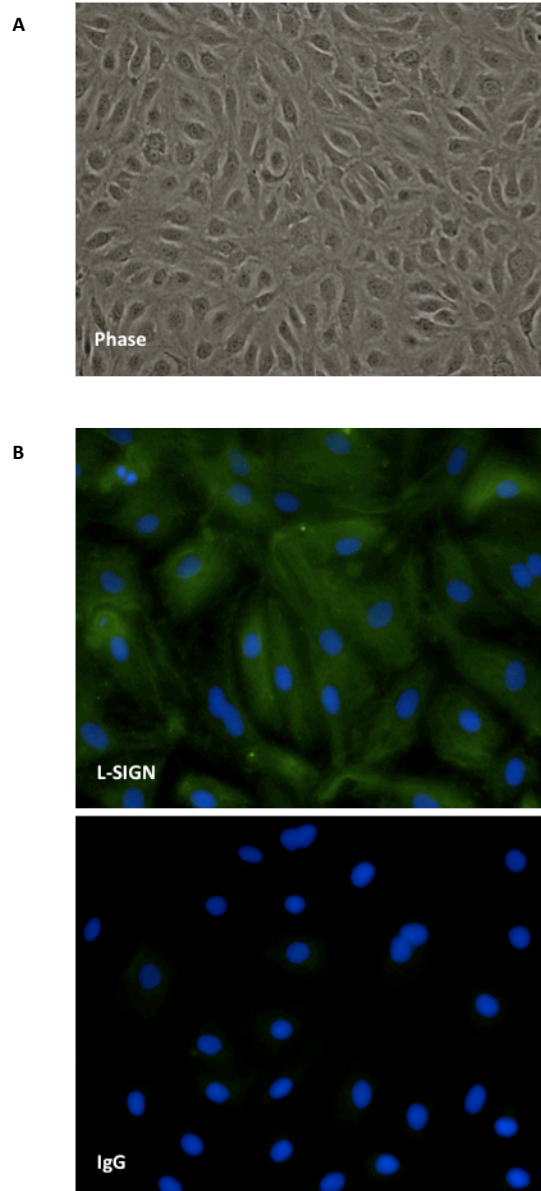


Figure 3-1. LSEC retain endothelial cell morphology and express characteristic phenotypic marker L-SIGN *in vitro*.

LSEC were propagated under standard culture conditions and fixed for phase contrast microscopy (magnification x100) (**A**). Cells were stained for L-SIGN as indicated (magnification x200) (**B**).

HCV entry into permissive cells is dependent on four critical entry factors: CD81, scavenger receptor BI (SR-BI), claudin-1, and occludin. Isolated LSEC were studied to determine the expression of these four critical entry receptors using both quantitative RT-PCR (**Fig. 3-2A**), and flow cytometry (**Fig. 3-2B**). LSEC, as previously described, expressed high levels of CD81, low levels of SR-BI whilst claudin-1 was not detected (Reynolds et al., 2008). However since that report occludin was described as a critical factor for entry and LSEC express low levels of this entry factor (Fletcher et al., 2012). In summary these data show that LSEC do not express the full complement of HCV receptors, predicting that LSEC are non-permissive for HCV entry and subsequent infection.

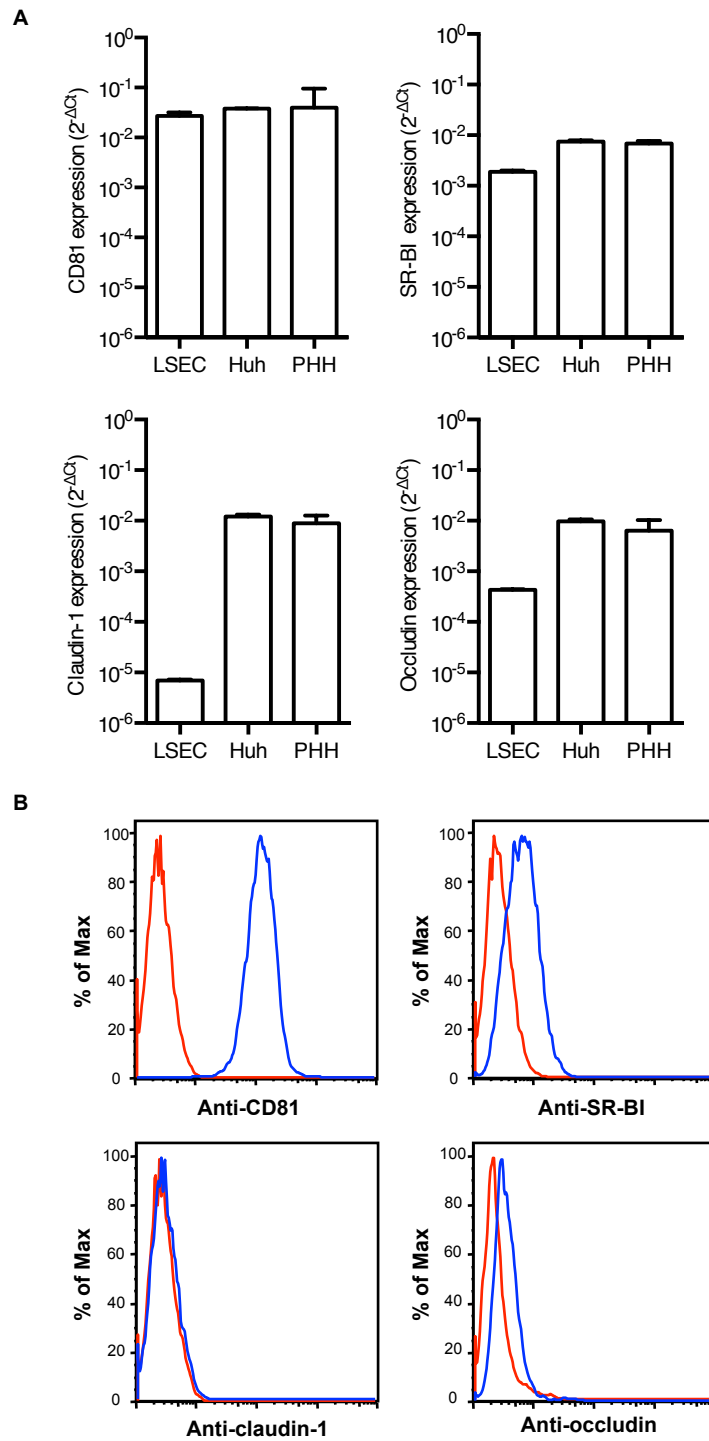


Figure 3-2. HCV receptor expression in LSEC.

Expression of HCV receptors CD81, SR-BI, claudin-1, and occludin was assessed by quantitative RT-PCR in LSEC, Huh-7.5 cells and primary human hepatocytes (PHH) (A). Gene expression is shown relative to expression of GAPDH using the $2^{-\Delta C_t}$ method (n=3 LSEC and PHH donors). Flow cytometric analysis of HCV receptor expression (B). The blue histogram shows receptor expression and the red histogram illustrates an irrelevant isotype control.

3.2 LSEC do not support HCV infection

Previous studies have suggested that LSEC are capable of binding infectious HCV virions due to their expression of HCV capture receptors CD81 and SR-BI. However there is limited evidence to confirm that LSEC associate with infectious virus particles in this way. To ascertain whether LSEC did indeed interact with infectious virions, LSEC were incubated with HCVcc for 2 hours and unbound virus removed by washing. Cell bound virus was measured by quantitative RT-PCR analysis of HCV RNA genome copies. Huh-7.5 hepatoma cells were included as a positive control and non-human Chinese hamster ovary (CHO) cell line as a non-permissive control cell type. HCV binding was observed at similar levels between LSEC and the permissive Huh-7.5 cell line and, more surprisingly, I also observed comparable binding to the CHO cells (**Fig. 3-3A**). This binding was possibly due to non-specific interactions and to ascertain HCV receptor dependency of binding CHO cells were transduced to express human SR-BI (Grove et al., 2007). When HCVcc was incubated with CHO cells or CHO-SR-BI cells comparable levels of virus bound to both the parental and transduced CHO cells (**Fig. 3-3B**). This suggested the possibility that non-specific interactions rather than receptor dependent binding govern initial interactions with infectious HCV *in vivo*.

Following the observation that LSEC can bind infectious HCVcc I was interested to know whether LSEC support HCV entry and replication. Screening for HCV entry

using the HCVpp system I was unable to demonstrate productive entry into LSEC (**Fig. 3-3C**). In keeping with this finding I was unable to detect HCVcc infection of LSEC using either the genotype 2a strain JFH-1 (**Fig. 3-3D**) and the chimeric genotype 1 strain J6/JFH-1 (data not shown). Infectivity for the Huh-7.5 cell line was confirmed with approximately 100,000 focus forming units (FFU) per mL of inoculum, demonstrating that LSEC do not support HCV infection *in vitro*.

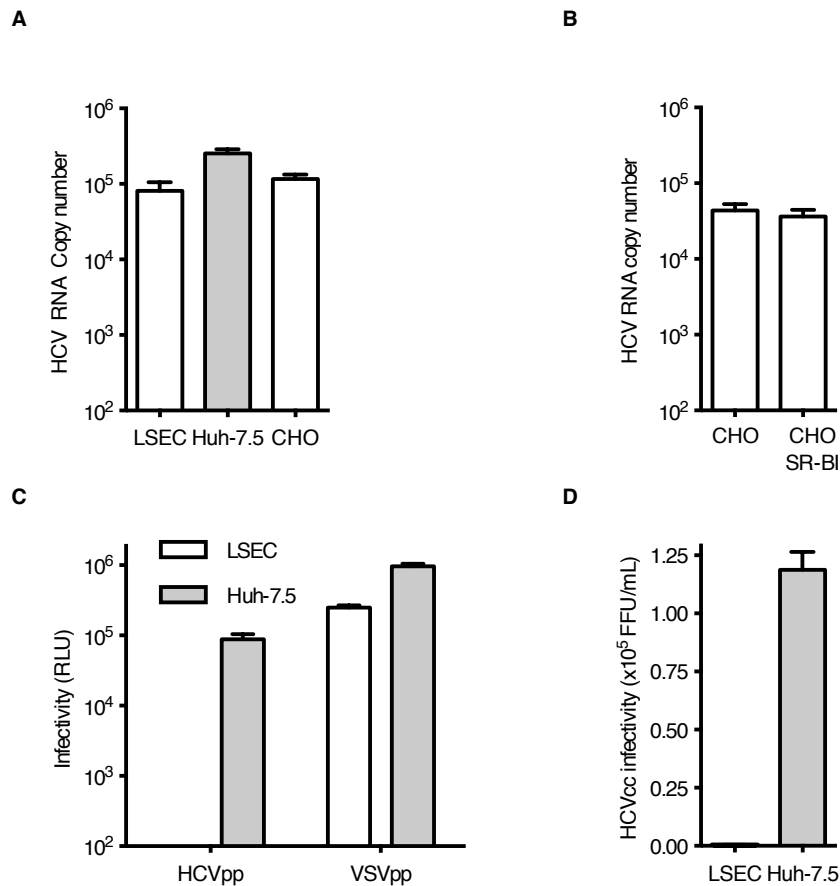


Figure 3-3. LSEC are non-permissive for HCV infection.

LSEC, Huh-7.5 cells, and Chinese Hamster Ovary (CHO) cells were incubated at 37°C for 2 hours with JFH-1 HCVcc, unbound virus was removed by washing and the cells lysed to measure HCV RNA by quantitative RT-PCR (n=3 LSEC donors) (**A**). Parental CHO cells and those transduced to express human SR-BI were treated identically and HCV RNA binding assessed by quantitative RT-PCR (n=2 independent experiments) (**B**). LSEC and Huh-7.5 cells were inoculated with HCVpp strain H77 and control VSV-Gpp luciferase reporter viruses for 8 hours, unbound virus removed and the cells maintained in culture for 72 hours. Luciferase activity was measured and the data expressed as relative light units (RLU) (n=4 donor LSEC) (**C**). In replicate experiments LSEC and Huh-7.5 cells were incubated with HCVcc JFH-1 for 4 hours and the cultures maintained for 72 hours (n=4 experiments with donor LSEC) (**D**). Cells were fixed and infection assessed by staining for viral antigen NS5A. Infectivity was defined as focus forming units (FFU) per mL of inoculum.

3.3 LSEC *trans*-infection of Huh-7.5 is inefficient

The current literature supports a role for LSEC to capture HCV particles from portal blood and to concentrate virus in the sinusoids, thus permitting contact with (and infection of) permissive hepatocytes (Protzer et al., 2012). It has also been suggested that LSEC actively transfer infectious virus to hepatocytes, defined as *trans*-infection, as previously reported for duck hepatitis B virus (Breiner et al., 2001). To assess this potential role for LSEC in promoting HCV infectivity I adapted a model successfully used to demonstrate HCV *trans*-infection by B lymphocytes (Stamatakis et al., 2009). LSEC were incubated with HCVcc JFH-1 for two hours, unbound virus was removed by washing and fluorescent labelled Huh-7.5 hepatoma cells added to achieve a confluent culture. The culture was maintained for 72 hours, fixed, and the cells stained for HCV antigen NS5A (**Fig. 3-4A**). As a control CHO cells that we previously demonstrated bound HCVcc at comparable levels to LSEC were included.

In initial experiments the cells were seeded at a 1:1 ratio and I noted that transmission events were rare in the LSEC containing co-cultures. When the cells were mixed each cell type separated out such that there were distinct islands of labelled Huh-7.5 cells surrounded by LSEC. In an attempt to promote viral transmission events the ratio of LSEC to Huh-7.5 was increased to 2:1 to provide a greater contact area between LSEC and Huh-7.5 cells. Using this approach there was a small increase in *trans*-infection events but these events were rare (**Fig. 3-4B**).

Indeed in comparison to cell-free HCV infectivity, LSEC *trans*-infection was at least 1000-fold less efficient. These data suggest that LSEC do not have a major role in potentiating HCV infection of permissive hepatocytes.

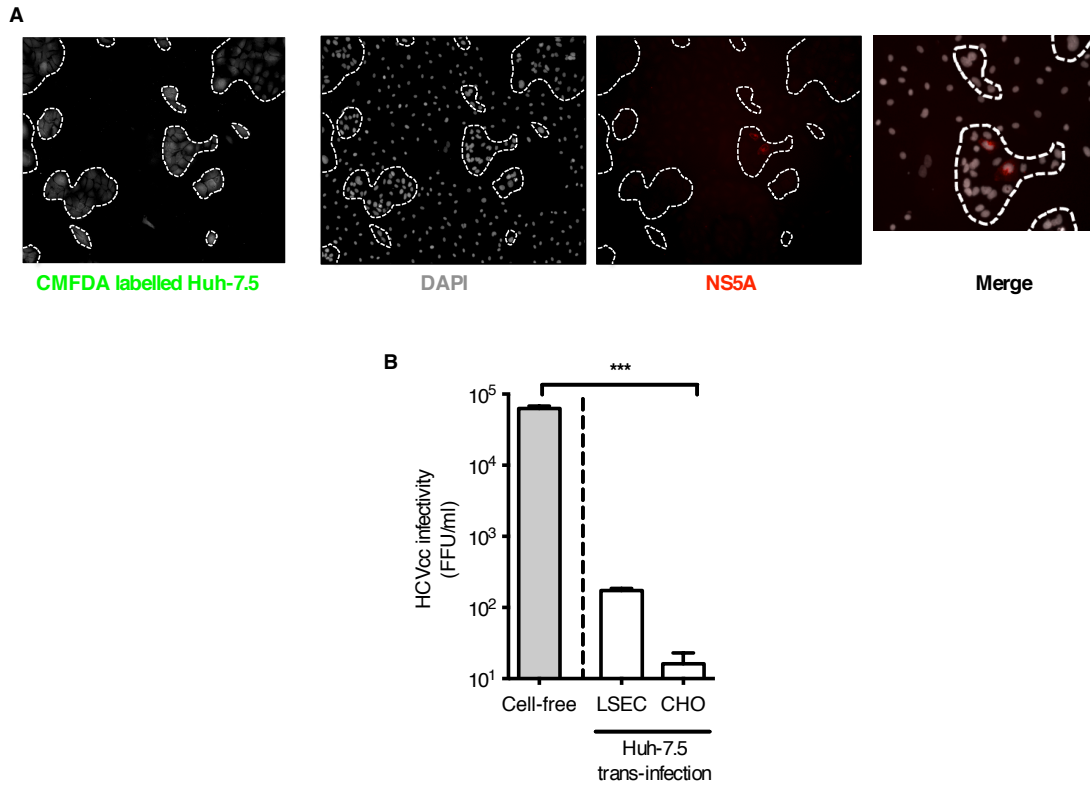


Figure 3-4. LSEC ineffeicently *trans*-infect Huh-7.5 cells.

LSEC and CHO cells were incubated with HCVcc JFH-1 (concentration 100,000 FFU/ml) for 2 hours at 37°C and washed extensively. CMFDA (cell-tracker green) labelled Huh-7.5 cells were added at a ratio of 1 Huh-7.5 cell to 2 LSEC and the culture maintained for 72 hours. Cells were fixed and stained for HCV antigen NS5A (red). Islands of Huh-7.5 cells are identified by the dotted line (**A**). Infectivity of cell bound HCV was quantified by enumerating NS5A expressing cells (**B**). Infectivity is expressed as FFU per mL of inoculum. Statistical comparisons were made with the Kruskal-Wallis test where *** $P < 0.001$ as indicated ($n=5$ donor LSEC).

3.4 Clearance of HCV RNA from the plasma after liver transplantation

HCV-related liver disease is one of the leading indications for liver transplantation in the western world (Brown, 2005). Following implantation of the allograft there is inevitable infection of the graft with HCV in the plasma at the time of surgery. Monitoring changes in peripheral HCV RNA during this period affords the opportunity to study virus clearance. As part of an on-going clinical trial of a small molecule inhibitor of SR-BI (clinicaltrials.gov NCT01292824) HCV RNA was monitored in the plasma of untreated patients to understand viral clearance kinetics.

During chronic infection there is some variability in the viral load of patients with end-stage liver disease (**Fig. 3-5A**). At the time of liver transplantation however I observed profound changes in plasma HCV RNA (**Fig. 3-5B**). During the operation blood supply to the diseased liver is interrupted signifying the onset of the anhepatic (literally “no liver”) phase. During this time there is no blood flow through the liver and, since the liver is the site of HCV replication, no HCV RNA enters the plasma from the liver. A slow decline in viral load was observed in the majority of participants (**Fig. 3-5B&C**) suggesting that HCV is relatively unstable in the periphery.

Following implantation of the allograft a second pattern was observed (**Fig. 3-5B&C**). In all participants there was a sharp fall in plasma viral RNA levels by approximately

90% within 1 hour of perfusing the new liver. This represents clearance of approximately 10^8 RNA genomes during this period. Such rapid clearance suggests the involvement of specialised systems and the kinetics are consistent with the liver being the major site of clearance. One of the primary roles of LSEC is to clear macromolecules from the portal circulation (Elvevold et al., 2008), lending further support for a protective role of LSEC in HCV infection.

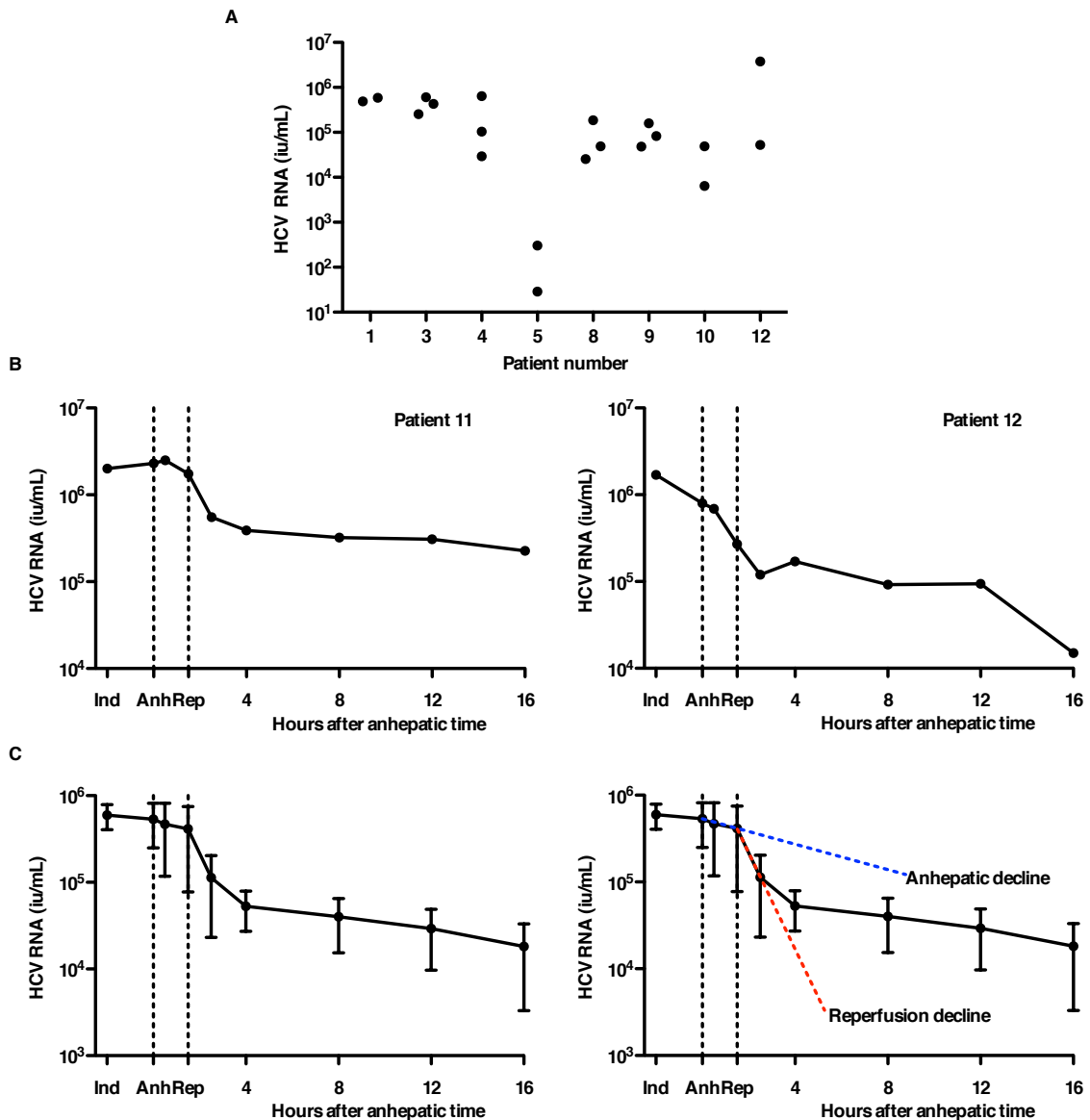


Figure 3-5. HCV is cleared rapidly from the plasma during liver transplantation.

HCV RNA levels were measured in patients undergoing liver transplantation. In the recruitment phase patients had blood sampled for HCV RNA quantitation during screening and on any subsequent admission for potential liver transplantation (**A**). After admission for liver transplant samples were taken at induction of anaesthesia (Ind), when the blood flow into the liver was clamped and the patient became anhepatic (Anh), and immediately before the new liver was perfused with blood (reperfusion – Rep). Additional samples were taken at intervening timepoints to indicate clearance rates as indicated (**B&C**). Data are mean \pm SD for n=13 patients (**C**).

3.5 LSEC reduce HCVcc infectivity in co-culture

The observations that LSEC *trans*-infection of hepatocytes is inefficient and that LSEC are likely to play a major role in clearing HCV from the circulating blood raised the question as to whether LSEC exert additional protective effects. To address this question I established co-cultures of LSEC and Huh-7.5 cells at a ratio of 1:1. Co-cultures were seeded to give a confluent culture after overnight incubation to limit the effects of differential growth kinetics. As previously described each cell type separated into distinct islands that were readily identifiable due to their differing phenotypic characteristics (**Fig. 3-6A**).

When co-cultures were infected with HCVcc for 48 hours I noted a substantial reduction in the infectious titres compared to Huh-7.5 cell mono-culture. Indeed in LSEC containing co-cultures infectivity was reduced by more than 80% (**Fig. 3-6B**). Co-culturing LSEC with Huh-7.5 cells in a 1:1 ratio reduced the number of permissive target cells by 50% however the reduction in infectivity mediated by LSEC was always greater than this. These findings suggest that any reduction in permissive cell number (or cell surface area) that would predictably reduce HCV infectivity is unlikely to completely explain the effect of LSEC in co-culture.

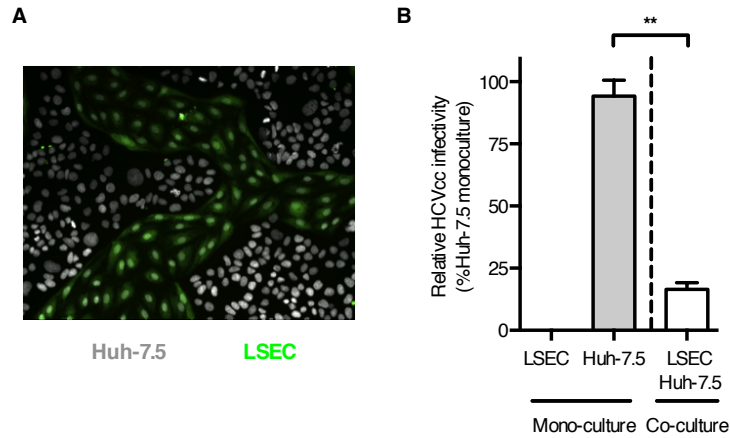


Figure 3-6. LSEC limit HCV infectivity in co-culture.

LSEC and Huh-7.5 cells were co-cultured at a ratio of 1:1 and infected with HCVcc JFH-1. LSEC were labelled with CMFDA (cell tracker green) to discriminate between cell types (A). Infectivity was determined by staining for HCV antigen NS5A and enumerating positive foci. Infectivity is expressed relative to Huh-7.5 monoculture. Statistical comparison was made using the Mann-Witney U test where ** $P < 0.01$ as indicated (n=4 donor LSEC).

3.6 Heterotypic cell interactions limit HCV infectivity

The observation that LSEC limit HCVcc infectivity highlighted a number of questions regarding the influence of non-permissive cells in the liver during HCV infection. To address these issues I established both LSEC and CHO cell co-cultures at increasing ratios of Huh-7.5 cell to non-permissive cell (**Fig. 3-7A&B**). The aim of these experiments was to address whether the number of permissive target cells in the co-culture would alter infectivity. A positive association between infectivity and the number of permissive cells was noted in the co-culture. However, in both conditions the number of infected foci in the co-culture is less than predicted by considering a Huh-7.5 monoculture seeded at a similar density (as indicated by the dotted line in **Fig. 3-7C**).

There are several reports on the effect of heterotypic cell interactions on hepatocyte function *ex vivo*. The mechanisms underlying these effects include cell contact, secreted factors and matrix derived signals (Bhatia et al., 1999, Hui and Bhatia, 2007, Khetani et al., 2004). To clarify the effect of LSEC and CHO cell contact in this experimental setting I used specially designed slides that allow cells to be cultured separately but still permit the sharing of soluble mediators. In these experiments I observed no effect of either LSEC or CHO cells on HCVcc infectivity (**Fig. 3.7D**) indicating that heterotypic cell contact is important in regulating HCVcc infectivity in the liver.

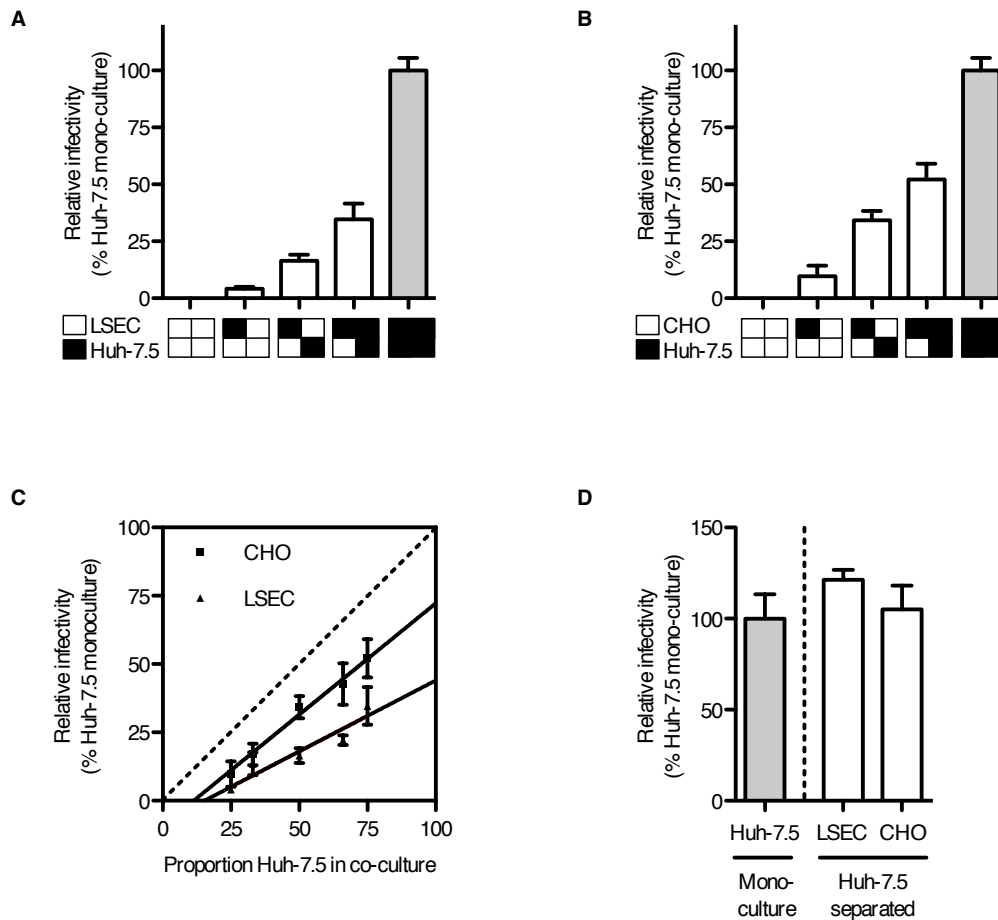


Figure 3-7. Heterotypic cell contact limits HCV infection.

LSEC Huh-7.5 cell (A), and CHO cell Huh-7.5 cell (B) co-cultures were established at varying ratios as indicated and infected with HCVcc JFH-1 at a multiplicity of infection of 0.01. Linear regression analysis indicated significant differences in the relationship between permissive cell number in the presence of LSEC or CHO cells. The dotted line indicates the hypothetical relationship in the absence of heterotypic cells (C). LSEC and CHO cells were separated from Huh-7.5 cells but maintained in media contact and infected with HCVcc as above (D). Infectivity was determined by staining for viral antigen NS5A and enumerating positive foci. Infectivity is expressed relative to Huh-7.5 monoculture (n=3 donor LSEC and CHO co-cultures).

3.7 Discussion

LSEC inefficiently transfer HCV to hepatocytes

Studying the role of primary human LSEC in the HCV life cycle has been hindered by difficulties in isolating these endothelial cells. However recent technical advances permit the study of this cell type *in vitro* (Liaskou et al., 2010, Shetty et al., 2011). Although isolated in relatively small numbers LSEC maintain typical morphology and phenotypic markers including L-SIGN until at least passage 4 in tissue culture. This has allowed these studies of this important cell type in co-culture systems as a model of the microenvironment of the liver lobule.

The role of cells in the liver other than hepatocytes in HCV infection has not been well documented. The liver is large and complex and it is likely that cells other than hepatocytes will impact on HCV replication (Protzer et al., 2012). We (Lai et al., 2006), and others (Cormier et al., 2004, Lozach et al., 2004), have suggested that LSEC capture and transmit infectious HCV particles to underlying permissive hepatocytes, suggesting a role for LSEC to potentiate chronic infection. The data presented here argue against this model and suggest that LSEC are protective in HCV infection.

As HCV enters the liver in portal (and arterial) blood it is distributed via the sinusoidal networks through the hepatic lobules. LSEC normally exert a scavenger function: taking up macromolecules for transcytosis to hepatocytes or destruction (Elvevold et al., 2008) and it has been hypothesised that HCV would be taken up in the same way (Protzer et al., 2012). Indeed LSEC are capable of binding HCV particles and although LSEC express both capture receptors for HCV, and the entry receptors CD81 and SR-BI, this binding is most likely to be through non-specific interactions with heparin sulphates or glycoaminoglycans. This hypothesis is supported by the observation that expression of high levels of SR-BI failed to significantly enhance HCV binding to CHO cells. Furthermore it has been reported that binding to heparin sulphates is a critical part of the initial attachment process and occurs before interaction with the specific receptors for HCV (Barth et al., 2006, Jiang et al., 2012). There are no reports of the functions of these attachment factors on LSEC, or indeed on any other non-permissive cell types. The specific infectivity of cell culture produced HCVcc is low (approximately 1/3000 particles is infectious (Lindenbach et al., 2006)) and receptor dependent binding is difficult to accurately quantify in non-permissive cells.

More importantly however is the functional significance of virus binding to LSEC, and this relates to the fate of virus particles bound. It has been shown in a duck hepatitis B virus model that LSEC actively transfer virus particles from sinusoidal blood to underlying hepatocytes (Breiner et al., 2001). To determine whether the

same may be true for HCV I adapted previously published protocols for lymphoid cell HCV *trans*-infection of permissive target cells (Stamatakis et al., 2009). In these experiments I saw very little transfer of infection from LSEC loaded with large quantities of virus. This suggests that most virus that is bound by LSEC is either not internalised or is targeted for destruction or antigen processing following uptake. Regardless, LSEC bound HCV particles are largely not infectious for Huh-7.5 hepatoma cells. Unfortunately since this process was so inefficient it was not possible to further study virus receptor interactions and tools to track virus, e.g. with fluorescently labelled virus, are not yet available. Therefore I am not able to define the pathways mediating these effects, nor am I able to define the fate of particles in LSEC.

It has been recently reported that LSEC are the critical cell type responsible for clearing blood-borne viral particles from the circulation (Ganesan et al., 2011). In patients with HCV infection and end-stage liver disease liver transplantation affords the opportunity of measuring viral “clearance” both in the presence and absence of liver blood flow (Dragun et al., 2011, Garcia-Retortillo et al., 2002, Powers et al., 2006). As part of a clinical trial of a small molecule inhibitor of SR-BI I have undertaken similar viral kinetic studies (clinicaltrials.gov NCT01292824). Our studies confirm a massive clearance of viral particles from the systemic circulation at the time of allograft perfusion that is most likely mediated by LSEC. One hour following perfusion approximately 90% of circulating RNA genomes have been cleared from

the circulation highlighting the capacity that exists for scavenger function in LSEC. The clearance of infectious pathogens is an important aspect of LSEC biology that would be worthy of further study in HCV infection when such tools are available. However, it seems most likely that hepatocytes interact directly with HCV particles in the sinusoidal space through LSEC fenestrations. Warren and co-workers (Warren et al., 2006) studied LSEC fenestrations by electron microscopy and noted that hepatocytes are able to protrude villi into the sinusoidal space where they likely contact circulating HCV particles. Thus in chronic infection LSEC are most likely to act as a sink for infectious particles protecting hepatocytes rather than supplying hepatocytes with infectious particles.

Heterotypic cell interactions limit HCV infection

Despite the complexity of the liver, there are relatively few studies on the role of non-parenchymal cells in HCV infection. To study the role of LSEC in HCV infection I established co-cultures of LSEC and Huh-7.5 cells. Significant reductions in viral infectivity in LSEC and CHO containing co-cultures were observed. I considered that simply reducing the surface area of permissive cells in the co-cultures would act to reduce HCVcc infectivity. For example, I reasoned that for a given duration of infection a reduction in permissive cell surface area would lead to a proportional reduction in infectivity since fewer infectious particles would have the opportunity to interact with a permissive cell. Indeed, I observed a positive association between virus infectivity and the proportion of Huh-7.5 cells in the co-culture. However,

infectivity was always reduced in co-culture when compared with that predicted by the proportion of permissive cells included. Since heterotypic cell contact has been reported to regulate hepatocellular function *in vitro* (Bhatia et al., 1999, Hui and Bhatia, 2007) I studied whether cell contact was essential to limit HCV infectivity in co-culture. Separating the cells but permitting exchange of soluble mediators restored infectivity to the levels seen in Huh-7.5 cell monoculture and thus heterotypic cell contact is essential to limit HCV infectivity.

The function of hepatocytes *in vitro* has been reported to be maintained by various methods of co-culture with supporting cells, both physiologically relevant (e.g. LSEC (Goulet et al., 1988)), or irrelevant (e.g. mouse fibroblast cell line 3T3 (Khetani and Bhatia, 2008)). The mechanisms that regulate changes in hepatocellular phenotype have been extensively investigated but remain elusive. It has been postulated that cell contact, matrix derived signals, and soluble mediators are all important players, however it is apparent that of these cell contact is absolutely required (Hui and Bhatia, 2007). I propose that these contacts maintain Huh-7.5 cells in a state that is less favourable for HCV infection. The literature suggests a role for supporting cells to stimulate a more hepatocytic phenotype. In this circumstance expression of cellular factors that govern HCV replication may be modulated to levels comparable to those seen in less permissive primary hepatocyte populations.

The study of HCV replication in primary human hepatocytes is complicated by limited availability of cells and variability between donors and cell isolation protocols. Following isolation hepatocytes have a limited lifespan of up to only 10 days since there is rapid dedifferentiation in culture, and HCV replication is only supported at low levels (Farquhar and McKeating, 2008). Thus whilst it is ideal to study HCV replication in the environment in which it occurs *in vivo*, studying HCV replication in primary human hepatocytes is difficult, and may be misleading. To improve this line of investigation human hepatocytes have been co-cultured on micropatterned culture plates with 3T3 fibroblasts (Ploss et al., 2010). The rationale for these studies was that the human hepatocytes would maintain their phenotype in the presence of the 3T3 cells for longer and would remain permissive for HCV infection. Indeed the authors observed greater permissivity to HCVcc in the co-cultures and suggested their use in drug screening and toxicological investigation. These results superficially appear contradictory to our studies: on one hand cell contacts between LSEC and Huh-7.5 cells reduce HCV infectivity, whilst on the other co-culture of 3T3 cells with primary human hepatocytes maintains permissivity. However the starting point for each experimental system is very different and I suggest that both systems argue the same point: heterotypic cell interactions maintain hepatocytic phenotype thus regulating HCV replication. *In vivo* hepatocytes are permissive for HCV and this is lost following isolation and culture. 3T3 cells maintain isolated hepatocytes in a state that is more in keeping with the *in vivo* state, and therefore maintain permissivity. In contrast Huh-7.5 cells are highly permissive (probably more so than hepatocytes *in vivo*) and co-culturing with LSEC establishes a more hepatocytic

phenotype thus reducing permissivity. Furthermore physiologically relevant cell contacts between LSEC and Huh-7.5 cells more potently reduced HCV infectivity than those contacts between CHO cells and Huh-7.5 cells suggesting additional effects of LSEC in our systems that have not been previously evaluated.

In summary I show that primary human LSEC bind HCV but are not permissive for infection. However, LSEC do not efficiently mediate *trans*-infection of permissive hepatoma suggesting that this is not their major function in HCV infection. In co-culture with Huh-7.5 cells LSEC reduced HCV infectivity in a cell contact dependent manner and I propose that the major role of LSEC in HCV infection may be protective rather than potentiating infection as previously suggested.

Chapter 4 Vascular endothelial growth factor signalling in liver sinusoidal endothelial cells limits hepatitis C virus replication

Paracrine signalling systems in the liver are critical for both the development and function of the organ. The foremost signalling system in the adult is regulated by hepatocyte derived vascular endothelial growth factor (VEGF-A) (Carpenter et al., 2005, DeLeve et al., 2004, Yamane et al., 1994). VEGF-A signals in sinusoidal endothelial cells to maintain their function in the normal healthy liver and following injury it stimulates the expression of hepatotrophic growth factors (Ding et al., 2010, LeCouter et al., 2003, Greene et al., 2003).

During HCV infection the expression of many soluble inflammatory mediators is increased. Many of these have been described in the context of inflammatory cell recruitment to the liver, the best described being the interferon- γ inducible chemokine CXCL10 (Asselah et al., 2005, Harvey et al., 2003, Zeremski et al., 2008). In addition, factors implicated in liver repair including VEGF-A have been reported to be upregulated (Hassan et al., 2009). It was noted that this increase in VEGF-A expression was accompanied by the activation of endothelial cells in the liver as evidenced by the proliferation of these cells and the formation of new blood vessels (Garcia-Monzon et al., 1995, Fernandez et al., 2009). This neoangiogenesis is a common feature in liver pathology highlighting the important role of VEGF-A in the

host response to liver injury. LeCouter and co-workers reported that VEGF-A activation of sinusoidal endothelium was protective in a chemical injury murine model (LeCouter et al., 2003), leading the authors to hypothesise that the same may be true in viral liver disease but the lack of an immune competent small animal model supporting HCV replication has restricted studies in this area.

The aims of this study were to characterise the role of VEGF-A in LSEC mediated effect(s) on hepatocellular HCV replication. I exploited the co-culture system described in Chapter 3 to investigate the effects of VEGF-A stimulation of LSEC and to determine whether this paracrine signalling was protective in HCV related liver injury.

4.1 LSEC express VEGF receptors and maintain responsiveness to VEGF-A

in vitro

Vascular endothelial cell responses to VEGF-A are mediated by two receptors: VEGF receptor-1 (VEGFR-1) and VEGF receptor-2 (VEGFR-2). LSEC expression of these receptors was confirmed by quantitative RT-PCR and western blotting (**Fig. 4-1A&B**). I observed high levels of *VEGFR-2* messenger RNA expression and significantly lower (approximately 100-fold less) *VEGFR-1* levels by quantitative RT-PCR. Protein analysis supported these conclusions indicating that VEGFR-2 is the major VEGF receptor expressed by LSEC.

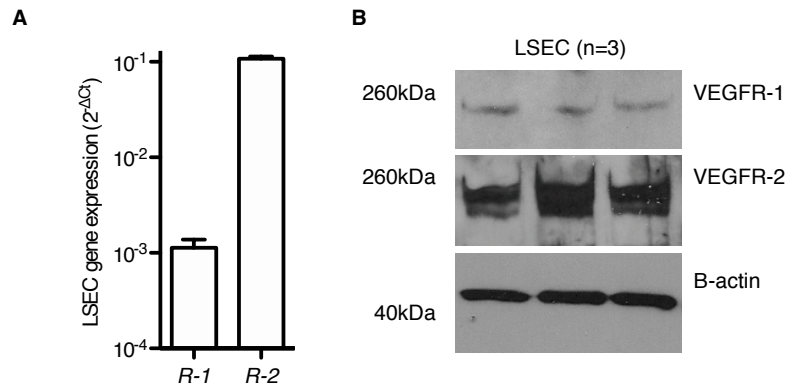


Figure 4-1. LSEC express VEGF receptors -1, and -2.

LSEC were cultured in standard conditions and lysed for quantitative RT-PCR analysis (**A**), and western blotting (**B**) (n=3 independent donor LSEC).

To determine whether these receptors were functional I examined both receptor phosphorylation and the LSEC proliferative response to recombinant VEGF-A. VEGFR-2 phosphorylation was readily detected in LSEC following VEGF-A stimulation (**Fig. 4-2A**). In parallel a dose-dependent increase in LSEC proliferation was observed (**Fig. 4-2B**) confirming that LSEC are responsive to VEGF-A *in vitro*. I was interested to know whether there was any variability amongst different donor LSEC in their response to VEGF-A. Using LSEC isolated from six independent donors (one of each of cryptogenic cirrhosis, fulminant seronegative hepatitis, primary biliary cirrhosis, and autoimmune hepatitis, and two from uninvolved liver tissue at the time of resection for colorectal cancer metastasis) I observed significant variability in the cellular proliferative response to VEGF-A over a 72 hour period (**Fig. 4-2C**). To ascertain the durability of the response to VEGF-A treatment LSEC were treated for defined periods of time as depicted in **Fig. 4-2D**. The increase in cell number correlated with the duration of VEGF-A treatment, suggesting that constant VEGF-A stimulation is required for proliferative responses.

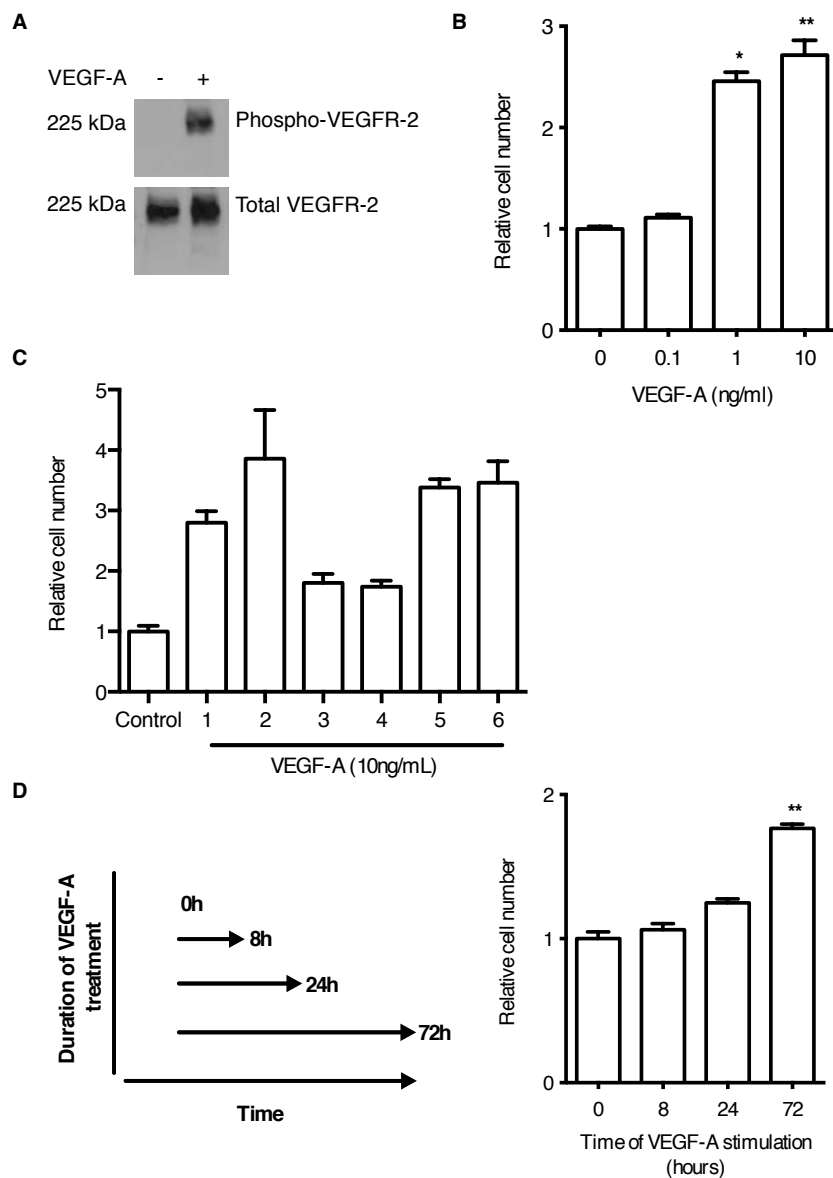


Figure 4-2. LSEC respond to VEGF-A *in vitro*.

LSEC were starved of VEGF-A overnight and treated with VEGF-A (10ng/mL) for 10 minutes. Cells were harvested for western blotting analysis (**A**). Following overnight VEGF-A starvation, LSEC were stimulated for 72 hours with VEGF-A at the doses indicated and proliferative responses assessed using MTS assay as described (n=4 donor LSEC) (**B**). LSEC from 6 independent donors (numbered 1 to 6) were treated with VEGF-A (10ng/mL) and proliferative responses characterised (**C**). Following overnight VEGF-A starvation LSEC were treated for different periods of time starting at time zero as indicated and proliferative responses assessed after 72 hours (n=4 donor LSEC) (**D**). Cell number is expressed relative to untreated control. Statistical comparison was made using the Kruskal-Wallis test and Dunn's correction where * $P < 0.05$, and ** $P < 0.01$ vs. untreated control.

To discern the receptor-dependency of the VEGF-A effects on LSEC proliferation I compared the efficacy of ligands targeting each of the two receptors in the presence or absence of receptor-specific neutralising antibodies. The ligands employed were VEGF-A that binds both VEGFR-1 and VEGFR-2, PlGF that is VEGFR-1 specific and VEGF-E targets only VEGFR-2. To validate the ligands and neutralising antibodies, VEGF receptor phosphorylation was confirmed following short duration treatment with VEGF-A (**Fig. 4-3A**). These studies confirmed specificity of VEGF-E and the neutralising antibody targeting VEGFR-2. I was unable to confirm similar activity for VEGFR-1 stimulation since receptor phosphorylation is weak and there are no antibodies available that detect endogenous levels of phosphorylated VEGFR-1 (Olsson et al., 2006).

LSEC proliferation was clearly mediated by VEGFR-2 with VEGF-E and VEGF-A stimulating comparable levels of cell proliferation (**Fig. 4-3B**). In addition, neutralising antibody targeting VEGFR-2 reduced proliferation in a dose dependent manner whilst anti-VEGFR-1 had no effect (**Fig. 4-3C**). Thus VEGFR-2 signalling is active in LSEC *in vitro* and this stimulates proliferation of this cell type.

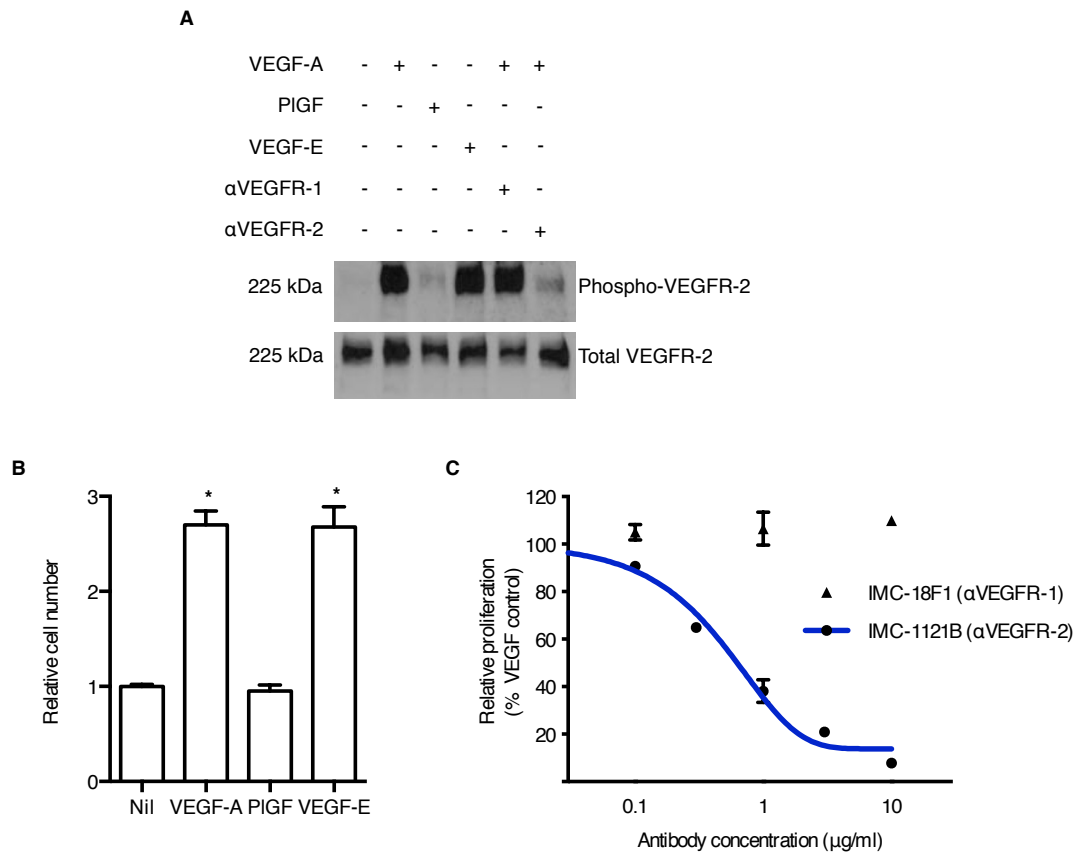


Figure 4-3. VEGFR-2 signals stimulate LSEC proliferation.

LSEC were starved of VEGF-A overnight, pretreated with receptor neutralising antibodies (α VEGFR-1 30 μ g/mL and α VEGFR-2 10 μ g/mL) for 1 hour, and then stimulated with VEGF-A (10ng/mL), PlGF (10ng/mL), or VEGF-E (10ng/mL) as indicated for 10 minutes. Cells were then harvested in lysis buffer for western blotting analysis (**A**). Following overnight VEGF-A starvation LSEC were stimulated for 72 hours with VEGF-A, or VEGF-E and proliferative responses of LSEC were assessed using MTS assay as described (n=6 donor LSEC) (**B**). Cell number is plotted relative to untreated control. LSEC were stimulated as above with VEGF-A after treatment with receptor neutralising antibodies. Cell number is plotted relative to VEGF-A stimulated control (**C**). Statistical comparison was made using the Kruskal-Wallis test and Dunn's correction where * $P < 0.05$ vs. untreated control.

4.2 Neutralising VEGF-A activation of LSEC increases HCV infectivity

VEGF-A stimulation of LSEC is critical in their response to liver injury and has been postulated to protect against viral infection. To ascertain whether VEGF-A plays a role in HCV infection of the LSEC Huh-7.5 co-culture system I infected the cells in the presence or absence of a neutralising anti-VEGF-A antibody. Neutralising VEGF-A promoted a significant increase in HCV infection of LSEC Huh-7.5 co-cultures to levels seen in control CHO Huh-7.5 co-cultures (**Fig. 4-4A**). In contrast, neutralisation of VEGF-A in these CHO cell containing co-cultures had no effect (data not shown). Importantly, the neutralizing anti-VEGF antibody had the same effect on viral infection of LSEC Huh-7.5 cultures when cells were seeded in Ibidi chamber slides and were not allowed to contact. Notably there was no effect when CHO cell containing co-cultures were treated with neutralising anti-VEGF-A antibody (**Fig. 4-4B**). Overall, these data suggest that activation of LSEC by hepatocyte derived VEGF-A may suppress expression of a proviral factor, or factors, thus limiting HCV infectivity.

To further define the role of VEGF-A activating LSEC in the co-culture model I infected the cells in the presence or absence of neutralising antibodies targeting VEGFR-1 and VEGFR-2 (**Fig. 4-4C**). Neutralising antibodies targeting VEGFR-2, rather than VEGFR-1, restored HCV infectivity back to the level of the CHO cell co-cultures suggesting that VEGF-A stimulation of LSEC via VEGFR-2 limits HCV infection.

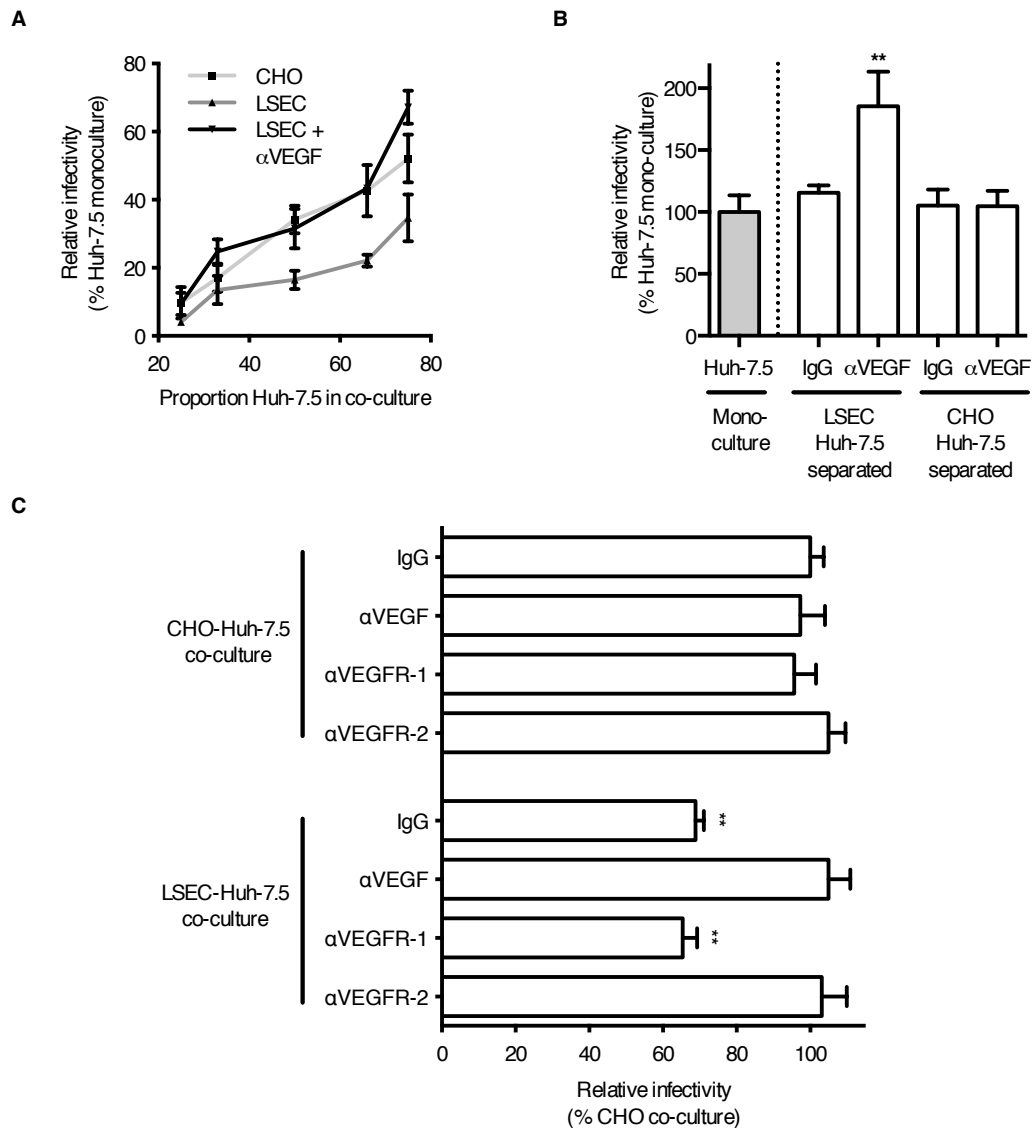


Figure 4-4. VEGF-A signalling reduces HCV infectivity in LSEC co-culture.

LSEC Huh-7.5 or CHO Huh-7.5 co-cultures were treated with neutralizing anti-VEGF-A antibody or irrelevant IgG (10µg/mL) prior to infecting with HCV JFH-1 (n=3 donor LSEC and for CHO co-cultures). **(A)**. Co-culture experiments were repeated where the different cell types were physically separated but allowed to share media **(B)**. Infectivity was determined by enumerating NS5A positive foci and is expressed relative to Huh-7.5 cell mono-culture. Co-cultures established at 1:1 ratios were treated with neutralising anti-VEGF-A antibody, or anti-VEGF receptor antibodies (αVEGFR-1 30µg/mL and αVEGFR-2 10µg/mL) and infected with HCV JFH-1 **(C)**. Infectivity is expressed relative to CHO cell containing co-cultures. Statistical comparison was made using the Kruskal-Wallis test and Dunn's correction where ** $P < 0.01$ vs. untreated control, or vs. CHO cell control co-culture (n=4 donor LSEC and for CHO co-cultures).

4.3 LSEC express factors that increase HCV replication in the absence of VEGF-A

Having observed that neutralising VEGF-A in LSEC Huh-7.5 co-cultures promoted viral infectivity I hypothesised that LSEC express soluble factors that potentiate infection. To investigate this further conditioned media from LSEC after incubation for 24 hours were collected, and used to treat Huh-7.5 cells prior to infecting with HCV. Using this approach I first treated Huh-7.5 cells with conditioned media from LSEC propagated in the absence of VEGF-A and observed a significant increase in HCV infection (**Fig. 4-5A**). I used this approach to determine whether the increase in infectivity was defined at the level of HCV replication. Following treatment of Huh-7.5 cells, either infected with HCVcc or expressing HCV replicons, with LSEC conditioned media similar increases in HCV RNA in cells infected with HCVcc and cells expressing full-length (and subgenomic, data not shown) HCV replicons was observed, confirming the stimulatory effect of LSEC on HCV replication (**Fig. 4-5B**). To determine whether VEGF-A activation of Huh-7.5 cells *per se* was responsible for modulation of HCVcc infectivity these cells were treated with recombinant VEGF-A prior to infecting with HCV JFH-1. No effect of VEGF-A stimulation was noted on HCVcc infectivity (**Fig. 4-5C**). I then treated LSEC with VEGF-A following overnight VEGF-A starvation and screened the conditioned media for its effect on Huh-7.5 permissivity to support HCV infection. A dose dependent decrease in HCVcc infectivity was noted (**Fig. 4-5D**), confirming that VEGF-A stimulation of LSEC is

responsible for modulating, and likely suppressing, expression of a soluble factor that regulates HCV replication.

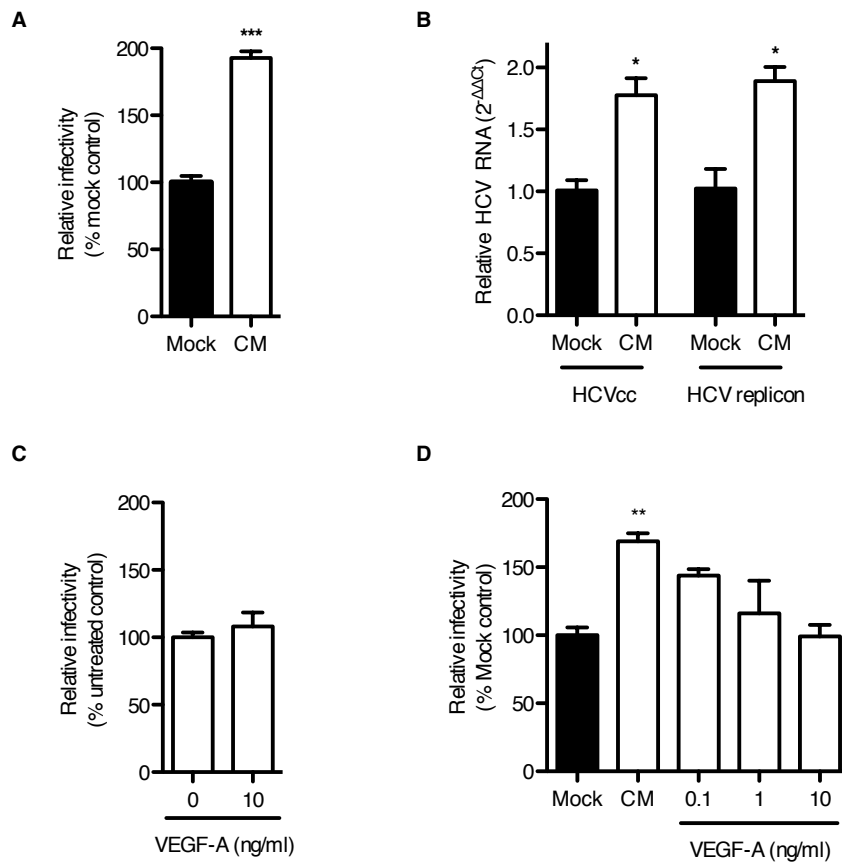


Figure 4-5. LSEC express proviral factor(s) in the absence of VEGF-A stimulation.

Conditioned media (CM) was collected from LSEC seeded at $4 \times 10^4/\text{cm}^2$ for 24 hours in the absence of VEGF-A. The media were diluted 1:2 with fresh media and used to treat Huh-7.5 cells for 18 hours before, and for 48 hours following infection with HCV JFH-1. Infection was enumerated by quantifying NS5A expressing cells (**A**) or HCV RNA levels (**B**) and the data expressed relative to untreated or mock Huh-7.5 cells. Huh-7.5 cells supporting JFH-1 replicons were treated with CM for 48 hours and HCV RNA levels measured (**B**). Recombinant VEGF-A (10ng/mL) was used to treat Huh-7.5 cells undergoing infection with HCV JFH-1 and infectivity assessed by enumerating NS5A positive foci (**C**). CM was collected from LSEC treated with increasing concentrations of recombinant VEGF-A and screened for its effect on Huh-7.5 permissivity to support HCV replication, as indicated above (**D**). Statistical comparisons were made with the Mann-Witney U test, or the Kruskal-Wallis test with Dunn's correction as appropriate and where * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, vs. Huh-7.5 monoculture or mock conditioned media as indicated ($n = 4$ donor LSEC conditioned media).

4.4 VEGFR-2 and p38 MAPK activation suppress pro-viral factor(s) in LSEC

VEGF-A signalling is complex and activation is mediated by two critical receptors, VEGFR-1 and VEGFR-2 (Olsson et al., 2006). The co-culture experiments have identified a key role for LSEC VEGFR-2 activation in regulating factors that promote HCV replication. Using the conditioned media systems I further investigated the pathways of LSEC activation.

First VEGF receptor specific ligands were used to confirm receptor dependence of the suppression of proviral factors in LSEC (**Fig. 4-6A**). Here I observed that signals activating VEGFR-2 (i.e. VEGF-E) suppressed HCVcc infectivity to comparable levels achieved with VEGF-A. This observation was confirmed with neutralising antibodies targeting the VEGF receptors (**Fig. 4-6B**). Neutralising anti-VEGFR-2 antibody treatment abrogated the VEGF-A mediated suppression of HCV infectivity whilst neutralising anti-VEGFR-1 antibody treatment had no effect.

I previously noted that constant VEGF-A stimulation was required for continued LSEC proliferation. LSEC conditioned media was therefore sequentially harvested every 24 hours for 3 successive days and used to treat Huh-7.5 cells prior to infecting with HCVcc (**Fig. 4-6C**). A similar suppression of HCVcc infectivity was observed in each of the conditioned media suggesting that LSEC do not become refractory to VEGF-A

stimulation of proviral factor expression and proliferative responses, both of which are regulated by activation of VEGFR-2.

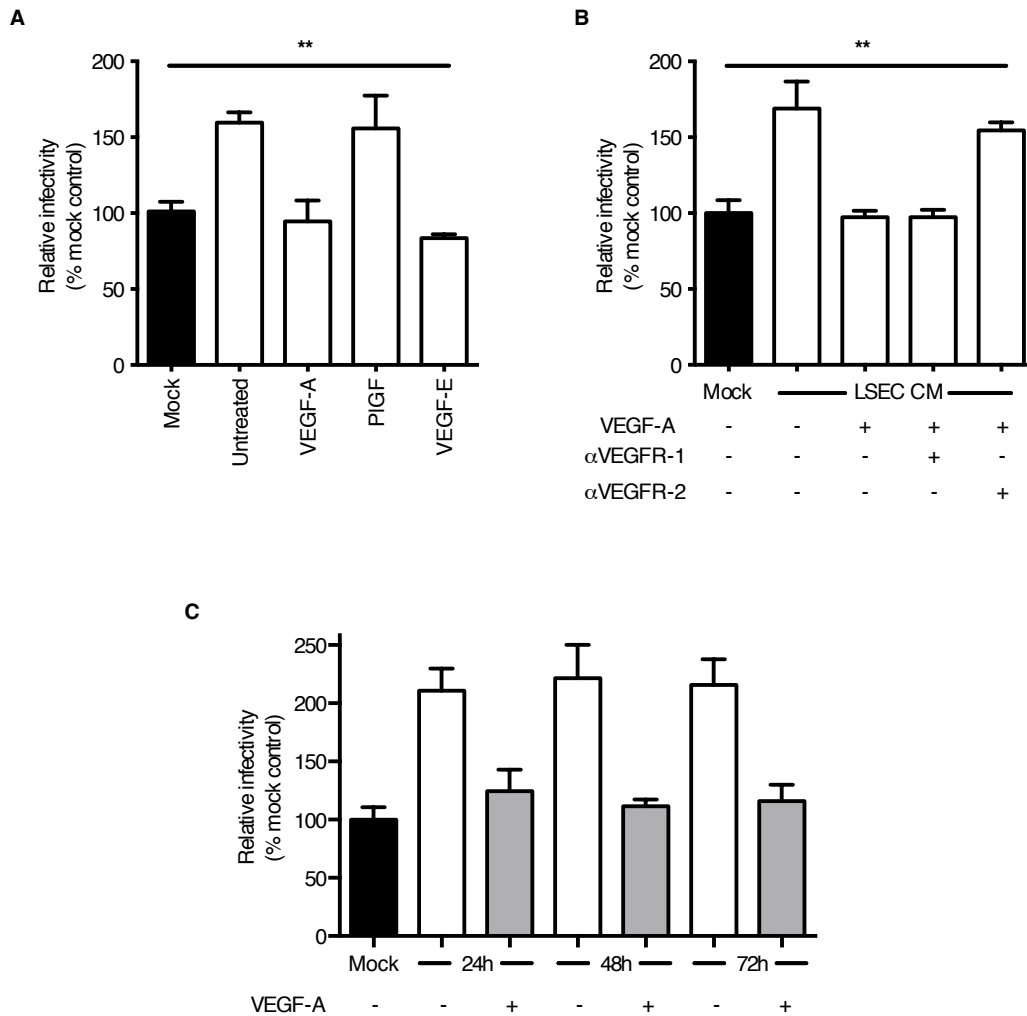


Figure 4-6. LSEC VEGFR-2 activation regulates proviral factor(s) expression.

Conditioned media (CM) were collected from LSEC seeded at $4 \times 10^4/\text{cm}^2$ for 24 hours that were unstimulated or treated with VEGF-A (10ng/mL). The media were diluted 1:2 with fresh media and used to treat Huh-7.5 cells for 18 hours before, and for 48 hours following infecting with HCV JFH-1 (**A**). Conditioned media were also collected from LSEC pre-treated with VEGF receptor neutralising antibodies (α VEGFR-1 30 μ g/mL and α VEGFR-2 10 μ g/mL) and subsequently stimulated with VEGF-A as indicated (**B**). Conditioned media were sequentially collected and used to treat Huh-7.5 cells as above (**C**). Infectivity was assessed by enumerating NS5A positive foci and is expressed relative to mock conditioned media control. Statistical comparisons were made with the the Kruskal-Wallis test and where ** $P < 0.01$ ($n=4$ donor LSEC conditioned media).

To characterise the intracellular signalling pathways downstream of VEGFR-2 I used various small molecule inhibitors of known pathways activated by this receptor (Olsson et al., 2006, Koch et al., 2011) (**Fig. 4-7A**). Specifically these inhibitors target MEK1 (PD98059), p38 MAPK (SB203580), phospholipase C (PLC, U73122), and PI3 kinase (wortmannin). When used at non-toxic concentrations only one inhibitor, SB203580, an inhibitor of p38 MAPK signalling abrogated the effect of VEGF-A on LSEC conditioned media (**Fig. 4-7B**). To ensure that p38 MAPK was activated by VEGFR-2 LSEC were treated with VEGF receptor specific ligands or with VEGF-A in the presence of specific neutralising antibodies (**Fig. 4-7C**). I noted that p38 MAPK was indeed activated by VEGFR-2 ligation and not VEGFR-1. LSEC were then treated with increasing doses of SB203580 and the conditioned media used to treat Huh-7.5 cells prior to infecting with HCV JFH-1 (**Fig 4-7D**). This experiment confirmed a dose dependent effect of p38 MAPK inhibition on VEGF-A priming LSEC to express soluble pro-viral factors. In summary these studies highlight a role for VEGFR-2 and p38 MAPK signalling in the regulation of endothelial expressed proviral factors.

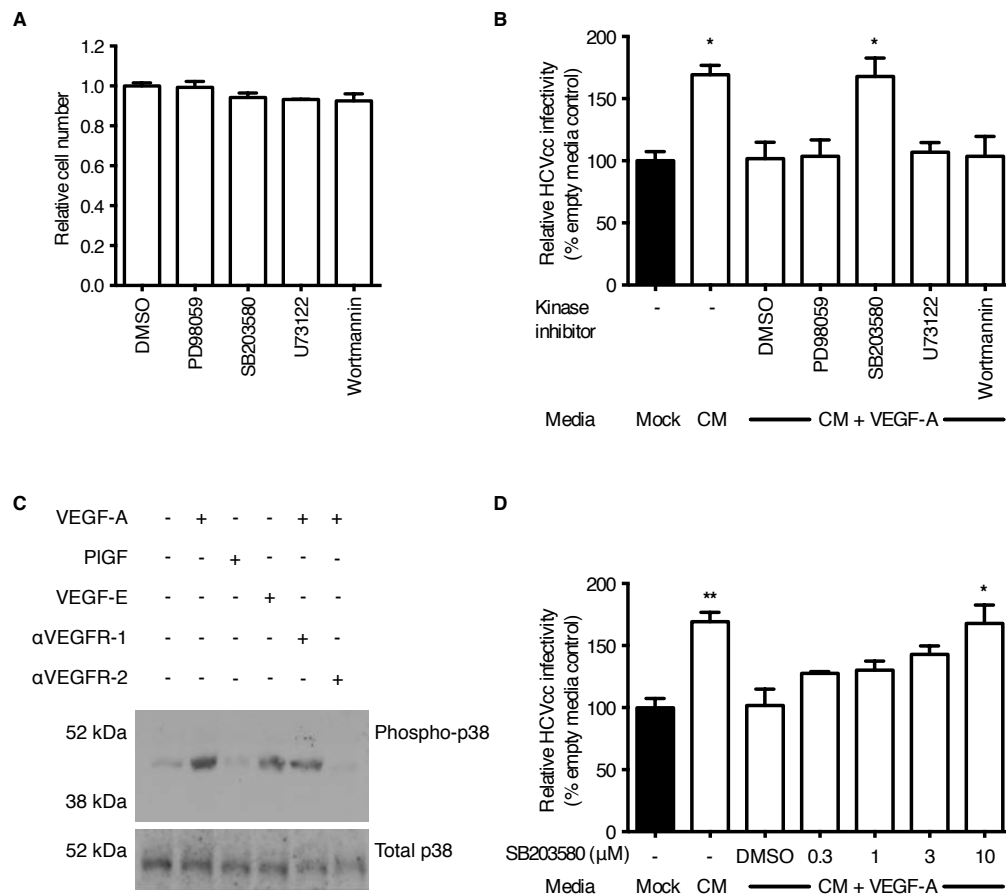


Figure 4-7. VEGF-A signals via p38 MAPK to suppress proviral signals.

LSEC were treated with chemical inhibitors (all 10μM) of pathways downstream of VEGFR-2 for 1 hour, washed and viability assessed 24 hours later by MTS assay (**A**). Conditioned media were then collected from LSEC seeded at $4 \times 10^4/\text{cm}^2$ pretreated for 1 hour with inhibitor or solvent control, and then stimulated with VEGF-A (10ng/mL) as indicated. The media were diluted 1:2 with fresh media and used to treat Huh-7.5 cells for 18 hours before, and for 48 hours following infection with HCV JFH-1 (**B**). LSEC were starved of VEGF-A overnight, pretreated with receptor neutralising antibodies (α VEGFR-1 30μg/mL and α VEGFR-2 10μg/mL) for 1 hour, and then stimulated with VEGF-A (10ng/mL), PIGF (10ng/mL), or VEGF-E (10ng/mL) as indicated for 10 minutes. Cells were then harvested for western blotting analysis (**C**). Conditioned media were collected from LSEC treated as above with increasing doses of SB203580 as indicated. Media were then used to treat Huh-7.5 cells as above (**D**). Infectivity was determined by enumerating NS5A positive foci and is expressed relative to mock conditioned media control. Statistical comparisons were made with the Kruskal-Wallis test with Dunn's correction and where * $P < 0.05$, and ** $P < 0.01$, vs. mock conditioned media ($n = 4$ donor LSEC conditioned media).

4.5 VEGF-A activation of large vessel endothelium limits HCV infectivity

LSEC are a highly specialised small vessel endothelial cell type that is phenotypically different from other endothelial cell types particularly large vessel endothelia (Lalor et al., 2006, Elvevold et al., 2008). I was therefore interested to know whether other endothelial cell types behaved in a similar fashion to LSEC in regulating HCV replication.

To investigate this human umbilical vein cells (HUVEC) were used as a model of large vessel endothelium. In replicate experiments LSEC and HUVEC were treated with VEGF-A and then following incubation for 24 hours harvested conditioned media that media was used to treat Huh-7.5 cells undergoing infection with HCVcc (**Fig. 4-8A&B**). I noted that in the absence of VEGF-A stimulation both LSEC and HUVEC increased HCVcc infection to comparable levels suggesting that expression of proviral soluble factors was conserved between these cell types. However, although VEGF-A regulated this soluble factor expression in HUVEC the VEGF-A stimulated reduction in HCV infectivity was consistently greater in LSEC than in HUVEC.

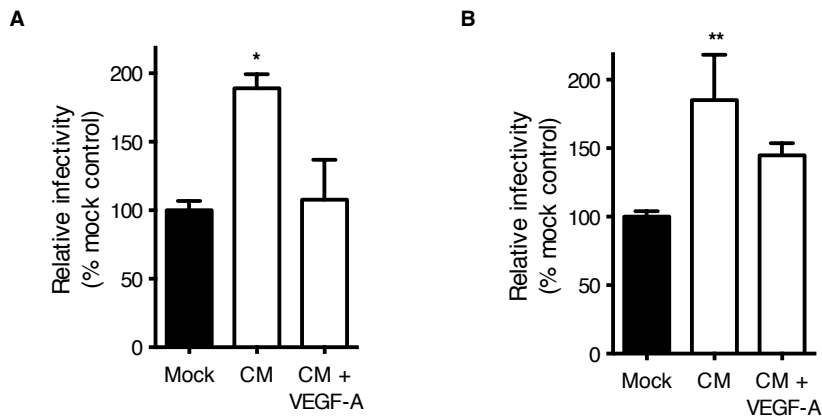


Figure 4-8. Large vessel endothelial cells express proviral factor(s) in the absence of VEGF-A stimulation.

Conditioned media were collected from LSEC (A), and HUVEC (B), each seeded at $4 \times 10^4/\text{cm}^2$. Cells were untreated, or were stimulated with VEGF-A for 24 hours as indicated. The media were diluted 1:2 with fresh media and used to treat Huh-7.5 cells for 18 hours before, and for 48 hours following infection with HCV JFH-1. Infectivity was assessed by enumerating NS5A positive foci and is expressed relative to the mock conditioned media control. Statistical comparisons were made with the the Kruskal-Wallis test with Dunn's correction and where * $P < 0.05$, and ** $P < 0.01$ vs. mock conditioned media (n=6 donor LSEC, and n=4 HUVEC conditioned media).

To begin to understand the mechanism underlying this difference I examined VEGF receptor expression in HUVEC and compared that to expression in LSEC. Measuring VEGF receptor expression by quantitative RT-PCR HUVEC expressed significantly more VEGFR-1, and less VEGFR-2 than LSEC (**Fig. 4-9A**). Furthermore the ratio of VEGFR-2 to VEGFR-1 was reduced in HUVEC suggesting that VEGFR-2 activation might be limited in HUVEC in response to VEGF stimulation. To characterise these differences further I examined VEGFR-2 phosphorylation by western blotting and observed lower phosphorylation in HUVEC with maximal VEGF-A stimulation (10ng/mL) in keeping with the reduced expression of total VEGFR-2 (**Fig. 4-9B**). Since VEGFR-1 may act as a decoy receptor for VEGF-A I examined the impact of the VEGFR-2 to VEGFR-1 ratio on the ability of VEGF stimulation of HUVEC and LSEC to suppress proviral factors. Using this approach the different cell types were readily segregated and there was a clear correlation between the VEGFR ratio and the suppressive ability of VEGF stimulation (**Fig. 4-9C**). These studies suggest that VEGFR-1 may be a limiting factor in VEGFR-2 activation and suppression of proviral factors in endothelial cells.

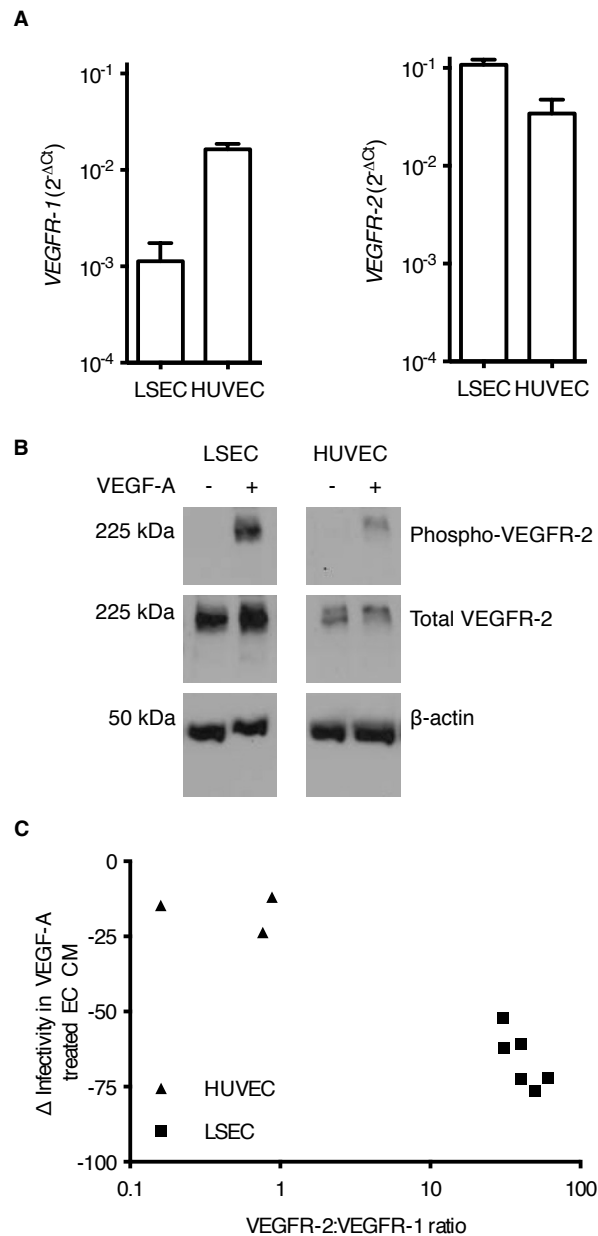


Figure 4-9. Increased levels of VEGFR-2 on LSEC are associated with increased suppression of endothelial proviral signals.

LSEC and HUVEC were maintained in standard culture conditions and VEGF receptor expression was assessed quantitative RT-PCR. Relative gene expression was calculated using the $2^{-\Delta C_t}$ method (n=3 donor LSEC and HUVEC) (A). Endothelial cells were starved of VEGF-A overnight and then stimulated with VEGF-A (10ng/mL) for 10 minutes. Cells were lysed for analysis by western blotting (B). The activity of VEGF-A stimulation was determined by the percentage decrease in HCV infectivity when stimulated conditioned media were compared to unstimulated media. This was then correlated with the relative abundance of VEGFR-2 to VEGFR-1 (n=6 donor LSEC and n=3 donor HUVEC) (C).

4.6 Discussion

LSEC VEGF receptor expression favours VEGFR-2 responses

VEGF-A is constitutively expressed by hepatocytes (Maharaj et al., 2006) and is the critical paracrine signal for LSEC *in vivo* that is responsible for normal endothelial cell function (Carpenter et al., 2005, DeLeve et al., 2004, Yamane et al., 1994). Here I show that VEGF-A stimulation of LSEC VEGFR-2 and p38 MAPK signalling is responsible for maintaining an environment that is less favourable for HCV infection due to the suppression of a soluble factor, or factors, that otherwise acts to increase viral replication.

The responses of endothelial cells in general to VEGF-A are well characterised and responses of isolated LSEC were explored as a facet of understanding the response to liver injury. I noted that the expression of VEGF receptors in LSEC was unusual compared with large vessel endothelium, and with published reports of VEGF receptor ratios in other endothelia (Jinnin et al., 2008). VEGFR-2 was expressed at significantly higher levels in LSEC and perhaps more importantly VEGFR-1 was expressed at significantly lower levels. The functions of VEGFR-1 have been variously described but the weight of evidence in normal development and tissue homeostasis supports a decoy function that protects against excessive activation of VEGFR-2 (Fischer et al., 2008). These findings together suggest that LSEC are primed

to respond to VEGFR-2 mediated signals. Crucially VEGFR-2 has been reported to maintain sinusoidal fenestrations and normal endothelial cell phenotype (Carpenter et al., 2005). Activation of VEGFR-2 was readily detected in LSEC exposed to VEGF-A or the VEGFR-2 specific ligand VEGF-E. Similarly LSEC proliferated in response to VEGF in a VEGFR-2 dependent manner. This response was limited to the duration of VEGF stimulation indicating both that continued stimulation (as is present in the liver (Maharaj et al., 2006)) is required for continued VEGFR-2 signalling, and that VEGFR-2 signalling is not refractory to repetitive stimulation. The unusual ratio of VEGFR-2 to VEGFR-1 seen in LSEC suggests that this phenotype is not beneficial in other endothelia. Indeed this is predictable since VEGF-A was first described as vascular permeability factor (Senger et al., 1983, Senger et al., 1986) and permeability would be deleterious in large vessels and in many other vascular beds, for example in the lung, or in the eye.

LSEC express pro-viral factor(s) in the absence of VEGF-A stimulation

The finding that in the absence of VEGF-A stimulation LSEC provided a proviral soluble signal was unexpected. LSEC had previously been reported to provide protective signals following VEGF-A stimulation in chemical liver injury leading the authors to hypothesise that the same may be true in viral infection (LeCouter et al., 2003). The observation that the effect of VEGF-A in the *in vitro* models was to suppress HCV infectivity following VEGFR-2 stimulation was broadly in line with that hypothesis. However, the protective effects of VEGF-A in the early rodent injury

models were reported to be mediated by VEGFR-1 signalling (LeCouter et al., 2003), however a later report studying liver regeneration failed to substantiate this and indicated a VEGFR-2 mediated mechanism (Ding et al., 2010). This further highlighted the importance of constitutive VEGFR-2 activation in human LSEC.

My first instinct when I noted VEGF-A stimulated suppression of HCV infectivity was to look for evidence of typical anti-viral effector molecules (interferon- α 2, interferon- γ , and tumour necrosis factor- α) in the LSEC culture media. No regulation of these factors was detected and in retrospect it was more likely that VEGF was directly regulating expression of a proviral molecule. To begin to characterise the likely effector molecule we screened pathways downstream of VEGFR-2 using chemical inhibitors (Olsson et al., 2006, Koch et al., 2011). Regulation of the putative proviral factor by p38 MAPK was identified. It is noteworthy that LSEC (and endothelial cells in general) have multiple roles in maintaining tissue architecture through expression of soluble mediators, termed 'angiocrine' factors (Butler et al., 2010). MAPKs have recently been implicated in this pathway (Kobayashi et al., 2010) and the observation of p38 MAPK involvement indicated biological plausibility to the VEGF-A regulated systems described.

In summary these data highlight a role for VEGF-A stimulated LSEC in maintaining an environment that is less permissive for HCV infection through suppression of a proviral factor, or factors. This occurs through VEGF stimulation of VEGFR-2 and p38

MAPK and illuminates a novel role for VEGF-A signalling and LSEC in chronic viral infection. To take these studies forward it was crucial to identify the soluble factor that is regulated by VEGF-A and to determine whether this factor is of pathophysiological importance in the liver injury associated with HCV infection.

Chapter 5 LSEC express bone morphogenetic protein 4 that promotes HCV replication

Since the development of cell culture methods to study HCV replication the majority of reports have focused on HCV infection of hepatocytes in isolation. Studying the relationship between different cell types in the liver will increase understanding of HCV pathogenesis and allow the development of new therapies targeting host cell pathways (Zeisel et al., 2012).

The studies on LSEC hepatocyte interactions had identified roles for both cell contact and VEGF-A dependent paracrine signalling to regulate HCV infection and to take these observations forward it was fundamental to identify the endothelial expressed soluble factor(s) that are regulated by VEGF-A. There are relatively few soluble factors reported to date that promote HCV replication and/or spread, for instance transforming growth factor- β (TGF β) (Lin et al., 2008, Lin et al., 2010) and epidermal growth factor (EGF) (Lupberger et al., 2011); however, neither of these factors are known to be regulated by VEGF-A. Furthermore, there were no data to support a role for LSEC in HCV infection above the previously suggested viral capture and transmission model.

The aim of this work was to identify the VEGF-regulated putative proviral factor expressed by LSEC using biochemical and transcriptomic analyses. Following identification of the soluble mediator I planned to study the importance of this molecule, or molecules in the pathogenesis of HCV infection.

5.1 Transcriptome analysis identifies genes regulated by VEGF-A in endothelial cells

I previously demonstrated that VEGF-A regulates LSEC expression of a putative proviral factor(s). Since similar responses were noted with HUVEC I decided to study the global gene expression in both LSEC and HUVEC in response to VEGF-A stimulation. In replicate conditions LSEC and HUVEC, from 2 independent donors, were cultured in the presence or absence of VEGF-A for 18 hours prior to lysis and RNA extraction for microarray analysis.

RNA quality was measured as outlined in the Materials and Methods and was used for 2-colour microarray analysis of gene expression. VEGF-A upregulated 273 genes >1.8-fold (with a false discovery rate of <5%) in LSEC and almost 500 genes in HUVEC. Importantly, only a minority of these genes (71) were upregulated in both cell types. A smaller number of VEGF down regulated genes were observed: 94 genes were down regulated in LSEC and 114 in HUVEC. Of these only 21 were regulated in both cell types (**Fig. 5-1A&B**).

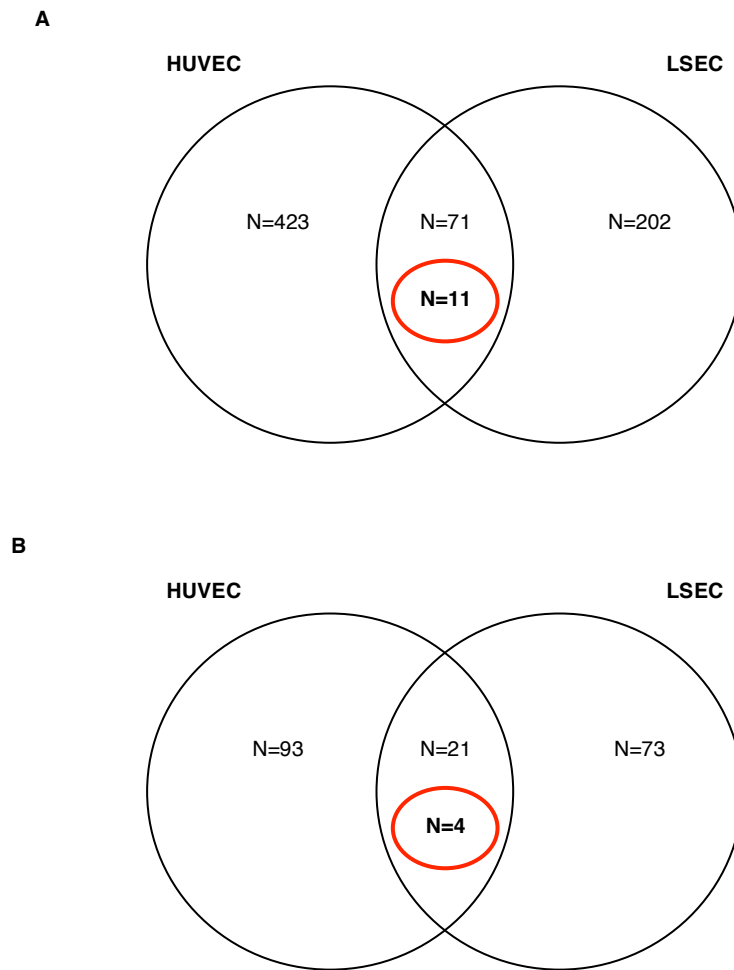


Figure 5-1. Transcriptomic analysis identifies a subset of genes regulated by VEGF-A in LSEC and HUVEC.

LSEC and HUVEC were starved of VEGF-A overnight and stimulated with VEGF-A (10ng/mL) for 18 hours. Cells were lysed for RNA extraction and microarray analysis. Genes up-regulated (**A**), or down-regulated (**B**) by VEGF-A were identified and overlapping regulation is shown in the Venn diagrams. Transcripts predicted to have a soluble signal product are highlighted in red circles.

Importantly endothelial expressed genes were enriched in the transcriptomic analysis. Amongst those transcripts identified to be regulated by VEGF-A were genes associated with angiogenesis (e.g. *CXCR4* (Salcedo et al., 1999, Salcedo et al., 2003) and *VEGFR-1* (Autiero et al., 2003, Fischer et al., 2008)), cell survival (*BCL2* (Reed et al., 1990, Alnemri et al., 1992)), and one that was characterised as a consequence of a previous cDNA microarray of VEGF-A regulated endothelial cell transcripts (*VASH* (Watanabe et al., 2004)).

To refine the search for transcripts encoding soluble molecules with pro-viral activity DAVID pathway analysis was employed (Huang da et al., 2009). This specialised bioinformatics resource allows the extraction of information from large quantities of biological information such as gene lists. The transcripts were classified by properties of the gene product, namely whether the product was predicted to be a soluble or paracrine signalling factor. Using this approach the number of candidate molecules identified in the transcriptomic analysis was reduced to 11 up-regulated genes and 4 down-regulated genes for further analysis (**Fig. 5-1**, and **Table 5-1**).

A.

Gene Symbol	Gene Name	LSEC FC	HUVEC FC
A2M	Alpha-2-macroglobulin	5.06	13.0
STAT	Statherin	4.78	4.07
PMCH	Pro-melanin concentrating hormone	4.68	5.07
CRLF1	Cytokine receptor-like factor 1	3.58	2.24
ADAMTS1	ADAM metalloproteinase with thrombospondin type 1 motif 1	3.37	2.31
FLT1	Fms-related tyrosine kinase 1 (VEGFR-1)	2.81	2.25
VASH	Vasohibin 1	2.69	1.85
MGP	Matrix gla protein	2.64	1.87
NID2	Nidogen 2	2.67	2.08
MMP1	Matrix metalloproteinase 1	2.47	2.36
PLAT	Plasminogen activator tissue	2.12	4.02

B.

Gene Symbol	Gene Name	LSEC FC	HUVEC FC
CXCL1	Chemokine (C-X-C motif) ligand 1	-4.74	-2.80
CCL2	Chemokine (C-C motif) ligand 2	-4.03	-3.85
CXCL2	Chemokine (C-X-C motif) ligand 2	-2.14	-1.86
BMP4	Bone morphogenetic protein 4	-2.11	-1.93

Table 5-1. Genes regulated by VEGF-A with predicted soluble product.

Genes with soluble product that were identified by DAVID pathway analysis as up-regulated (**A**), or down-regulated (**B**), in both endothelial cell types. FC, fold change.

To validate the microarray findings VEGF-A dependent regulation of selected genes in LSEC and HUVEC was confirmed from 2 additional donors (**Fig. 5-2**). These experiments showed consistent regulation of genes as predicted by the microarray. Importantly however, since none of the genes that had been identified as likely soluble signals had previously been implicated in HCV infection I sought to characterise the conditioned media biochemically to guide identification of the putative proviral factor.

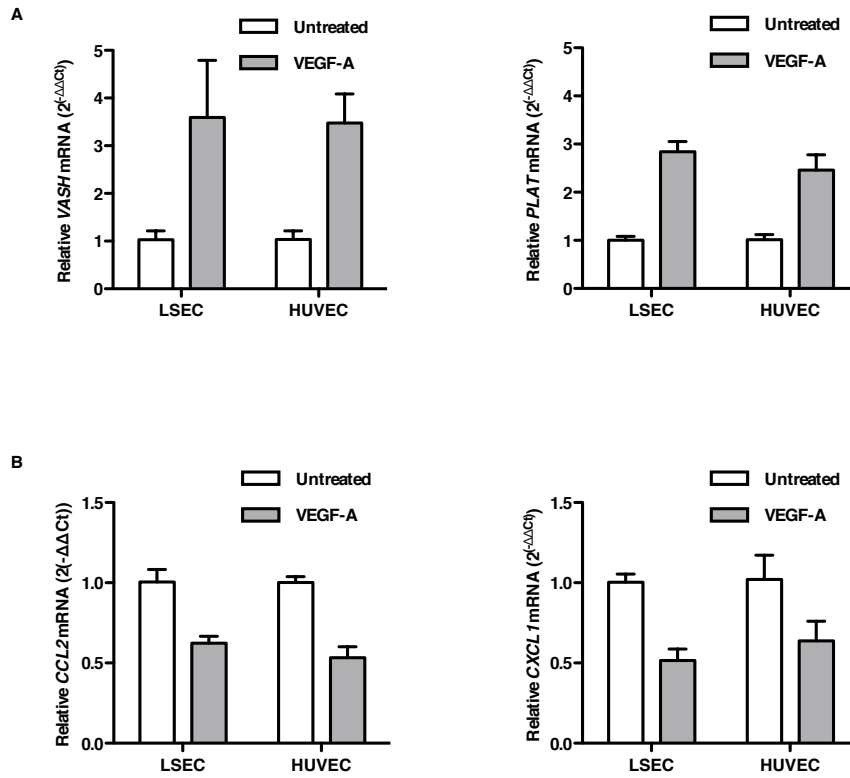


Figure 5-2. Validation of VEGF-A regulated transcripts.

Expression of predicted VEGF-A regulated transcripts was determined in LSEC and HUVEC from two independent donors. Endothelial cells were starved of VEGF-A overnight and stimulated with VEGF-A for 18 hours as indicated. Expression of predicted up-regulated genes (**A**), and down-regulated genes (**B**) was determined by quantitative RT-PCR. Expression is shown relative to untreated control cells using the $2^{-\Delta\Delta Ct}$ method.

5.2 Biochemical characterisation of LSEC expressed proviral factor(s)

The hypothesis that LSEC express a soluble pro-viral factor that is negatively regulated by VEGF-A is based on data showing that pre-treating hepatoma cells with endothelial cell conditioned media increased their permissivity to HCV infection. Initial experiments confirmed that conditioned media collected from LSEC isolated from 6 independent donors, in the presence or absence of VEGF-A, had similar effect(s) on HCV infectivity. I observed a consistent increase in HCV infection of hepatocytes treated with LSEC conditioned media that was ablated by VEGF-A (**Fig. 5-3A**). Importantly, the proviral activity of un-stimulated media showed a dose-dependent relationship following serial dilution of that media (**Fig. 5-3B**).

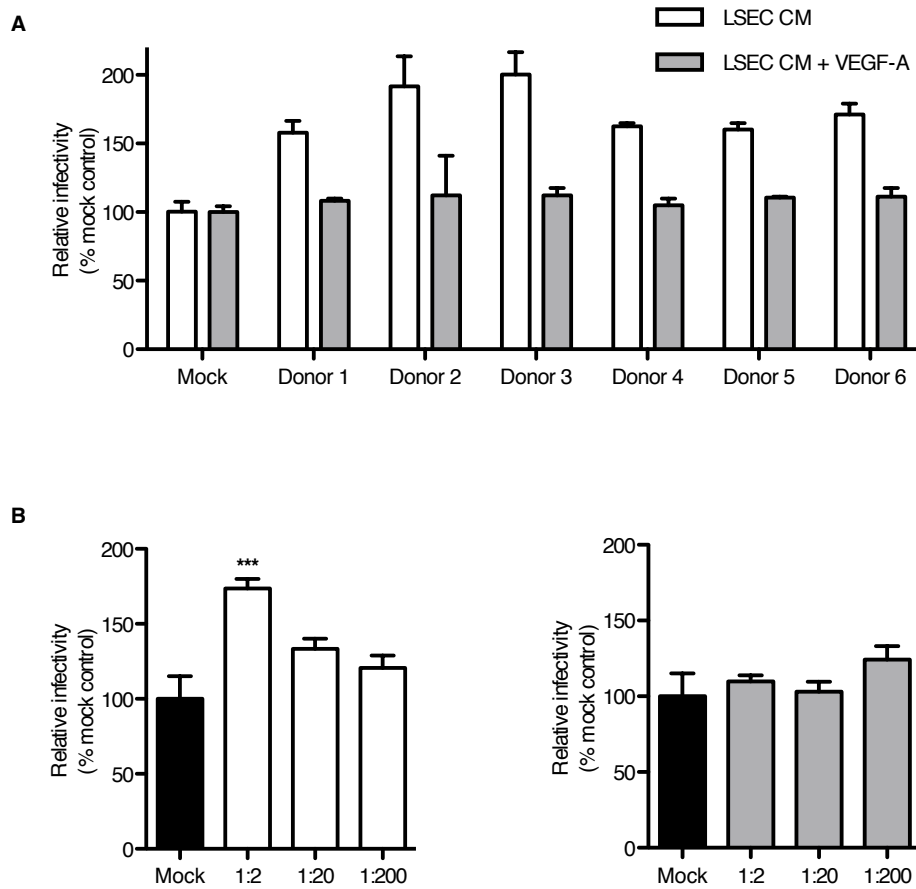


Figure 5-3. LSEC express proviral factors in the absence of VEGF-A stimulation.

Conditioned media collected from LSEC isolated from 6 independent donors seeded at $4 \times 10^4/\text{cm}^2$ either untreated, or stimulated with VEGF-A (10ng/mL) for 24 hours. The media were diluted 1:2 with fresh media and used to treat Huh-7.5 cells for 18 hours before, and for 48 hours following infection with HCV JFH-1 (**A**). Infectivity was assessed 48 hours post-infection by staining for NS5A and enumerating positive foci. Conditioned media were also diluted in fresh media as indicated before treating Huh-7.5 cells and infectivity assessed as above (**B**). Statistical comparisons were made with the Kruskal-Wallis test with Dunn's correction and where *** $P < 0.001$ vs. mock conditioned media ($n=6$ donor LSEC conditioned media).

Secondly the conditioned media were size-fractionated to identify the molecular weight of the putative pro-viral soluble factor(s) (**Fig. 5-4**). Using columns with 10, 30, 50, and 100 kDa size exclusion membranes I tested the ability of fractions to modulate HCV infection of Huh-7.5 cells. Molecular weight partitioning of non-stimulated conditioned media showed that the larger than 50 kDa fraction had no effect on HCV infection, whereas the fraction of at least 30 kDa in size was active, suggesting the pro-viral factor(s) were between 30 and 50 kDa in molecular weight (**Fig. 5-4A**). As expected, size fractionating the VEGF-A stimulated conditioned media had no effect of viral infectivity, suggesting that VEGF-A acts to suppress a proviral factor (**Fig. 5-4B**).

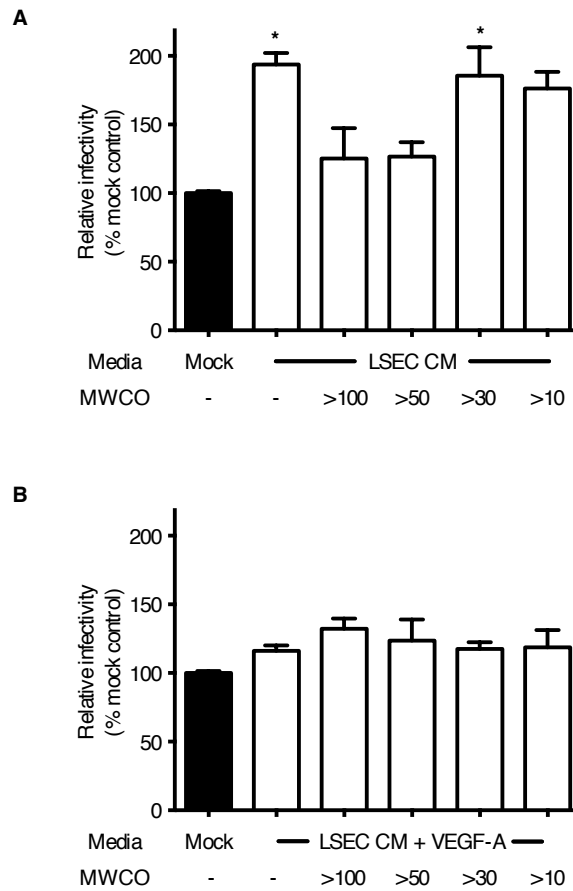


Figure 5-4. Molecular weight screening of LSEC proviral soluble factor(s).

Conditioned media (CM) was collected from LSEC in the absence (**A**) or presence of VEGF-A (10ng/mL) (**B**). Media was fractionated using molecular weight cut-off (MWCO) exclusion membranes. The media were diluted 1:2 with fresh media and used to treat Huh-7.5 cells for 18 hours before, and for 48 hours following infection with HCV JFH-1. Infectivity is presented relative to Huh-7.5 cells treated with non-conditioned media (black bars). Statistical comparisons were made with the Kruskal-Wallis test with Dunn's correction where * $P < 0.05$, and ** $P < 0.01$, vs. mock conditioned media (n=3 donor LSEC conditioned media).

5.3 Identification of BMP4 as a proviral factor in HCV infection

Using the data generated in the preceding experiments I reviewed the microarray data to identify soluble factor(s) of the defined molecular weight. Four genes (*CCL2*, *CXCL1*, *BMP4*, and *CXCL2*) were identified in the microarray that were down-regulated by VEGF-A. Of these only BMP4 has a molecular weight that is compatible with the size-fractionation experiments (36 kDa). The other molecules are chemokines with molecular weights of 11 kDa. Initial experiments confirmed that VEGF-A regulates LSEC expression of BMP4. Since LSEC are maintained in VEGF-A during routine culture to aid their proliferation *BMP4* gene expression following VEGF-A withdrawal was monitored (**Fig. 5-5A**). A significant increase in *BMP4* expression following 24 hours VEGF-A withdrawal was noted. I then treated LSEC starved of VEGF-A with recombinant growth factor and observed a dose dependent decrease in *BMP4* messenger RNA levels (**Fig. 5-5B**) that paralleled the effect of VEGF-A on pro-viral activity of LSEC conditioned media. In replicate experiments, LSEC were stimulated with VEGF-A and BMP4 protein expression determined by immunoblotting. Reduced BMP4 expression was noted consistent with results from the transcript analyses (**Fig. 5-5C**).

Finally I explored the pathway regulating the VEGF-A response in LSEC. Using VEGF receptor specific ligands I noted that VEGFR-2 activation suppresses BMP4 expression (**Fig. 5-5D**). Furthermore since a role for the p38 MAPK signalling

pathway had been observed in LSEC pro-viral activity (**Fig. 4-7**), this pathway was manipulated with a chemical inhibitor and monitored the effect on *BMP4* messenger RNA expression. Chemical inhibition of p38 MAPK dose dependently blocked the effect of VEGF-A stimulation on BMP4 expression (**Fig. 5-5E**). In summary, these studies show that LSEC expression of BMP4 is regulated by VEGF-A via a VEGFR-2 p38 MAPK dependent pathway.

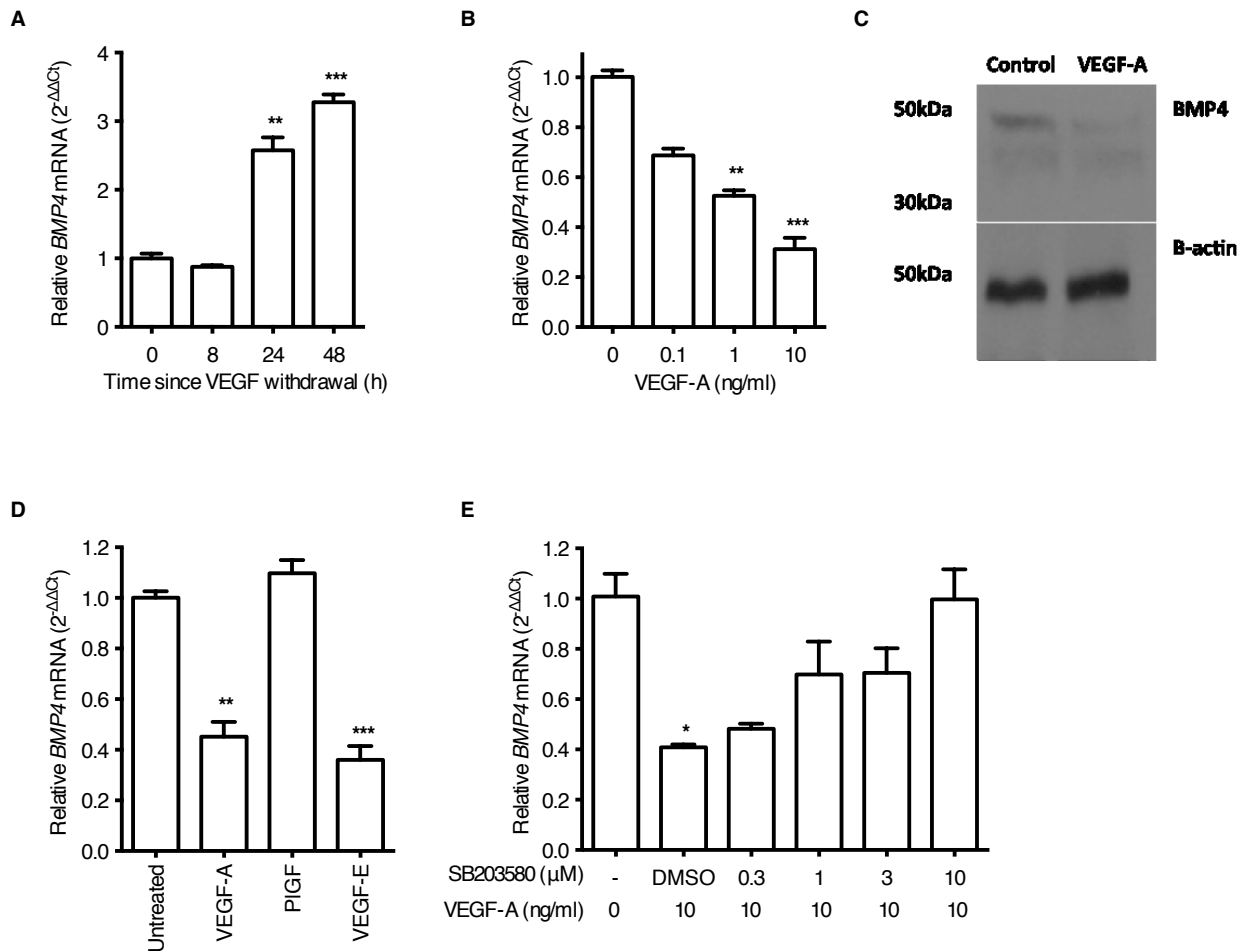


Figure 5-5. BMP4 is regulated by VEGF-A in LSEC.

LSEC were starved of VEGF-A stimulation for the time indicated and *BMP4* expression assessed by quantitative RT-PCR (**A**). Following overnight VEGF-A starvation LSEC were stimulated with VEGF-A (at the doses indicated) for 18 hours and *BMP4* expression assessed (**B**). *BMP4* expression is expressed relative to untreated control using the $2^{-\Delta\Delta C_t}$ method. In replicate experiments after 24 hours of VEGF-A (10ng/mL) stimulation LSEC were lysed to analyse protein expression by immunoblotting (**C**). LSEC were starved of VEGF-A overnight and stimulated with VEGF-A (10ng/mL), PlGF (10ng/mL), or VEGF-E (10ng/mL) as indicated for 18 hours before lysis and quantitative RT-PCR (**D**). Following VEGF-A starvation LSEC were treated with p38 MAPK inhibitor (SB203580) at the doses indicated for 1 hour before cells were washed and stimulated with VEGF-A (10ng/mL) for 18 hours. Expression of *BMP4* was assessed by quantitative RT-PCR (**E**). Statistical comparisons were made with the Kruskal-Wallis test with Dunn's correction where * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, vs. untreated control or mock conditioned media as indicated (n=6 donor LSEC for panel A-D, and n=3 donor LSEC for panel E).

To establish a role for BMP4 in HCV infection I used recombinant protein to study multiple aspects of the HCV lifecycle. Firstly I screened for an effect of BMP4 on HCVcc infection that recapitulates the entire lifecycle. A dose-dependent increase in HCV infection was noted in keeping with the hypothesis that BMP4 is a proviral factor (**Fig. 5-6A**). Importantly, the BMP4-dependent increase in HCV infectivity was also observed with primary human hepatocytes as the target cell for viral infection (**Fig. 5-6B**). I confirmed that BMP4 promotes HCV RNA replication in hepatoma cells supporting sub-genomic and full-length replicons (**Fig. 5-6C**). Finally the HCVpp system was used to screen for an effect of BMP4 on HCV glycoprotein-dependent entry. In these studies no effect of BMP4 on HCVpp entry into Huh-7.5 cells was noted confirming that the effect of BMP4 is to stimulate HCV replication and thus increase overall infectivity. These findings recapitulated the earlier observations with LSEC conditioned media, supporting a role for BMP4 in HCV replication.

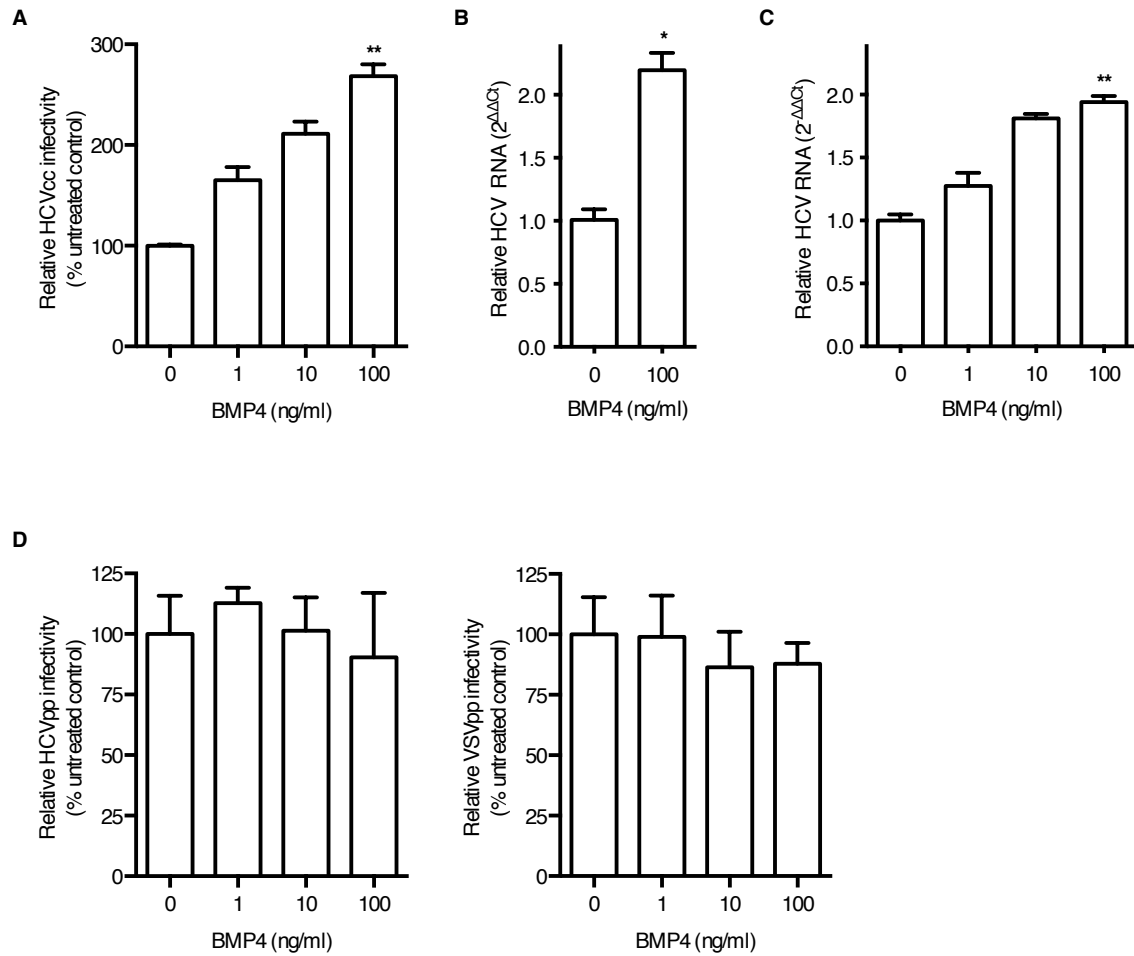


Figure 5-6. BMP4 increases HCV replication.

Huh-7.5 cells were treated with increasing doses of BMP4 and infected with HCV JFH-1. 48 hours post infection cells were stained for NS5A and infectivity assessed by enumerating NS5A positive foci (A). Primary human hepatocytes were treated with BMP4 (100ng/mL) and infected with HCV JFH-1. 72 hours post infection cells were lysed for quantification of HCV RNA abundance by quantitative RT-PCR (B). Huh-7.5 cells harbouring HCV subgenomic replicons were treated with increasing doses of BMP4 and 72 hours later were lysed for quantitative RT-PCR analysis (C). HCV RNA abundance is expressed relative to untreated control. Increasing doses of BMP4 were used to stimulate Huh-7.5 cells undergoing infection with HCVpp, or control VSVpp. After 72 hours cells were lysed for quantification of luciferase signal that is expressed relative to untreated control (D). Statistical comparisons were made with the Mann-Witney U test, or the Kruskal-Wallis test with Dunn's correction as appropriate where * $P < 0.05$, and ** $P < 0.01$, vs. untreated control ($n = 4$ independent experiments).

Finally to confirm a role for BMP4 in LSEC conditioned media on HCV infectivity I studied the effect of neutralising antibodies targeting BMP4 (**Fig. 5-7**). To confirm antibody specificity Huh-7.5 cells were treated with BMP4 in the presence or absence of anti-BMP4 neutralising antibody (**Fig. 5-7A**). These studies confirmed that anti-BMP4 blocked the effect of recombinant BMP4 on viral infectivity. These experiments also indicated that there was no contribution from BMP4 in Huh-7.5 monocultures, suggesting that the hepatoma cells do not express BMP4. Neutralising BMP4 abrogated the proviral effect of unstimulated LSEC conditioned media (**Fig. 5.7B**), confirming the role of BMP4 as the pro-viral factor expressed by LSEC that stimulates HCV infectivity in the absence of VEGF-A stimulation.

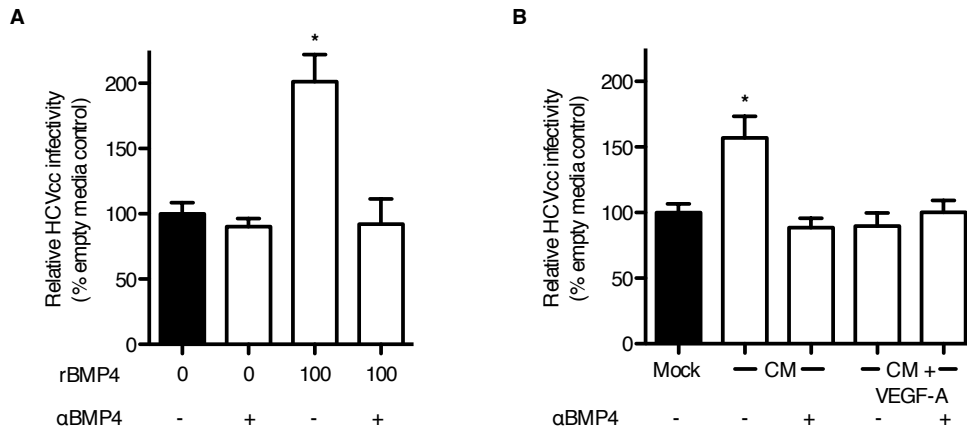


Figure 5-7. LSEC expressed BMP4 increases HCV infectivity.

Huh-7.5 cells were treated with BMP4 as indicated in the presence or absence of neutralising anti-BMP4 antibody (10µg/mL) (**A**). LSEC conditioned media were generated from cells treated with VEGF-A (10ng/mL) as previously described and were treated with neutralising anti-BMP4 antibody as indicated. The media were diluted 1:2 with fresh media and used to treat Huh-7.5 cells for 18 hours before, and for 48 hours following infection with HCV JFH-1 (**B**). Infectivity was assessed by enumerating NS5A positive foci. Statistical comparisons were made with the Kruskal-Wallis test with Dunn's correction where * $P < 0.05$, vs. untreated control, or mock conditioned media as indicated (panel A, $n=3$ experiments; panel B, $n=4$ donor LSEC conditioned media).

5.4 BMP4 and VEGFR-2 expression in human liver

There are limited reports on the role of BMP4 in adult liver and these relate largely to involvement in pathological processes including fibrosis (Fan et al., 2006) and carcinogenesis (Chiu et al., 2012, Maegdefrau et al., 2009, Maegdefrau and Bosserhoff, 2012). I therefore sought to investigate BMP4 and VEGFR-2 expression in normal and diseased liver.

VEGFR-2 expression localised to the liver sinusoidal endothelium (**Fig. 5-8A**), consistent with the quantitative RT-PCR detection of *VEGFR-2* messenger RNA in LSEC (**Fig. 5-8B**). It was not possible to confidently identify cell types in the liver expressing BMP4 by immunohistochemical methods however *BMP4* messenger RNA was largely restricted to LSEC *ex vivo* (**Fig. 5-8C**), suggesting that this cell type is a major site for expression in the liver.

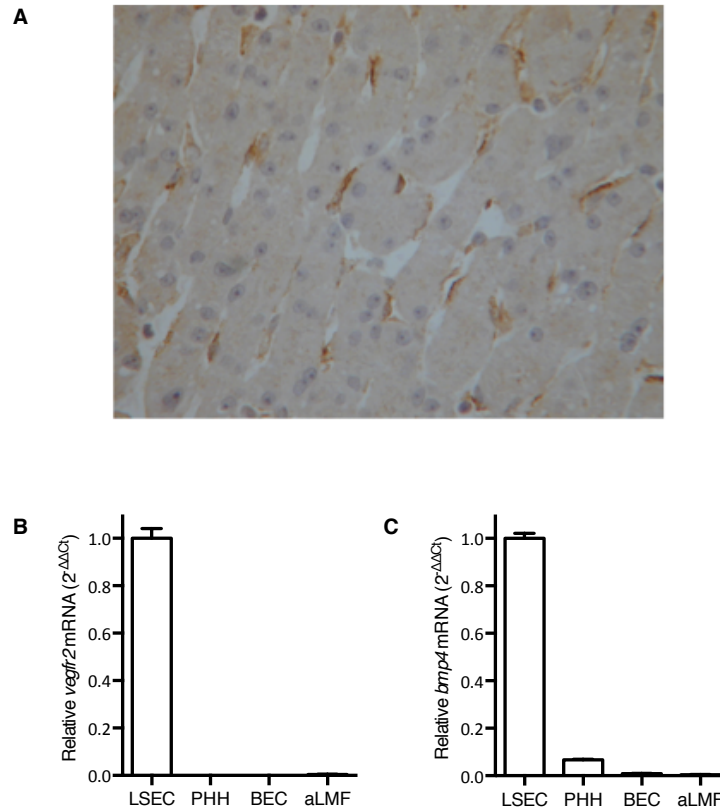


Figure 5-8. VEGFR-2 and BMP4 expression is largely restricted to LSEC.

Sections of normal human liver were stained with antibody specific for VEGFR-2 (**A**). Constituent cell types (LSEC; PHH, primary human hepatocytes; BEC, biliary epithelial cells; aLMF, activated liver myofibroblasts) were isolated from diseased liver and expression of *VEGFR-2* (**B**), and *BMP4* (**C**) was determined using quantitative RT-PCR. Gene expression is expressed relative to LSEC using the $2^{-\Delta\Delta C_t}$ method (n=3 donors for each cell type).

Finally before embarking on studies of HCV infected whole liver it was critical to determine whether HCV infection *per se* regulates expression of BMP4 in infected hepatocytes. Using Huh-7.5 cells infected with high titre HCVcc *BMP4* messenger RNA was quantified at 72 hours post infection and compared to uninfected cells. In cultures where approximately 80% of Huh-7.5 cells were infected I did not observe any increase in *BMP4* expression suggesting that HCV infection does not regulate BMP4 directly (**Fig. 5-9A**). Importantly *VEGF-A* messenger RNA expression was increased following HCV infection as previously reported (**Fig. 5-9B**). In summary, these studies highlight a role for LSEC as the major cell type in the liver expressing BMP4.

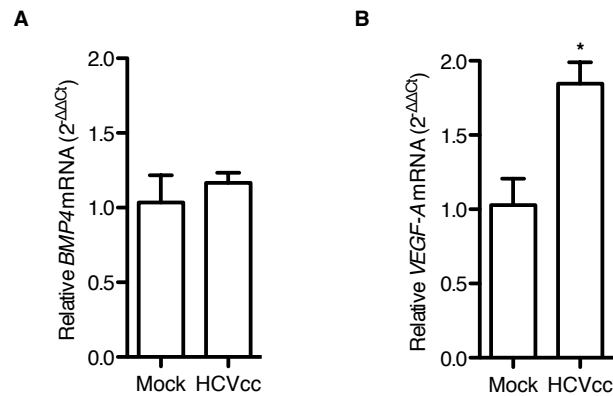


Figure 5-9. HCV infection does not regulate BMP4 messenger RNA expression.

Huh-7.5 cells were infected with high titre HCVcc JFH-1. 72 hours post infection the cells were lysed for analysis of gene expression by quantitative RT-PCR. Expression of *BMP4* (A), and *VEGF-A* (B) were quantified and expressed relative to the untreated control using the $2^{-\Delta\Delta C_t}$ method. Statistical comparisons were made with the Mann-Witney U test where * $P < 0.05$, vs. mock untreated control (n=4 independent experiments).

5.5 Regulation of BMP4 expression in HCV-associated liver disease

The role of BMP4 in liver disease is poorly defined and to determine the effects of HCV in the liver I studied human liver from patients with end-stage liver disease from either HCV related liver disease, or from alcohol related liver disease (ALD). These specimens were compared to normal liver that was surplus to requirements for transplantation purposes.

Using these samples I first looked at expression of *BMP4* messenger RNA and protein across the disease states. In these studies low expression of BMP4 in healthy liver and increased expression in the diseased tissue was noted. Notably expression was greatest in patients with ALD rather than in patients with HCV infection (**Fig. 5-10**).

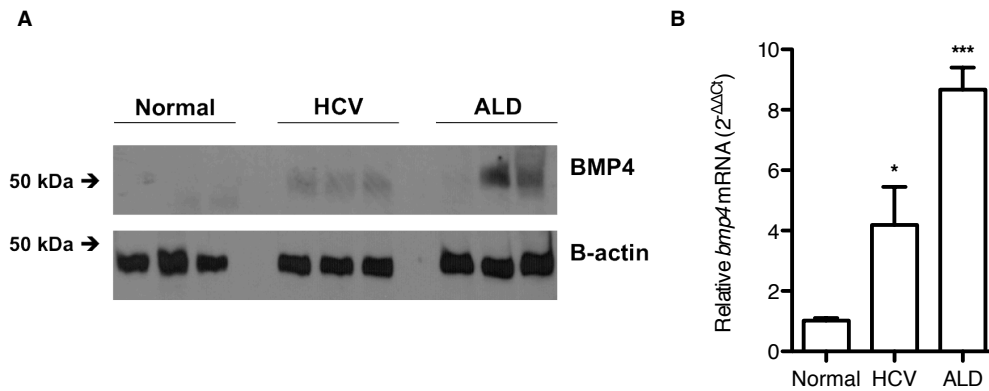


Figure 5-10. BMP4 expression is increased in chronic liver disease.

Samples from normal liver, and diseased liver as a consequence of HCV infection or alcohol related liver disease (ALD) were analysed for BMP4 protein (**A**) and messenger RNA expression (**B**). Representative blots from 3 donors are shown. *BMP4* expression is expressed relative to normal liver control using the $2^{-\Delta\Delta C_t}$ method. Statistical comparisons were made with the Kruskal-Wallis test with Dunn's correction where * $P < 0.05$, and *** $P < 0.001$, vs. normal liver control (n=6 for each disease type).

We, and others, have previously reported that HCV infection increases VEGF expression and the finding that BMP4 expression was increased in disease was surprising given that the *in vitro* studies would predict an inverse association between VEGF-A and BMP4 expression. I therefore studied the expression of VEGF-A and its cognate receptors VEGFR-1 and VEGFR-2 in the normal and diseased liver tissue samples (**Fig. 5-11**). Both VEGF-A and VEGFR-2 messenger RNA and protein were increased in HCV and ALD related liver disease (**Fig. 5-11**). Since a clear dependence of VEGFR-2 activation to suppress BMP4 mRNA levels had been observed, I studied VEGFR-2 phosphorylation as a marker of receptor activation (**Fig. 5-11D-E**). Interestingly VEGFR-2 expression was increased in liver disease where angiogenesis and increases in endothelial cell number are well recognised (Fernandez et al., 2009). However, there was little change in the levels of VEGFR-2 phosphorylation observed between normal and diseased liver samples. The resulting outcome is a reduction in the ratio of phosphorylated VEGFR-2 to total VEGFR-2 in diseased liver, and, by inference, a reduction in the VEGFR-2 activation on a per endothelial cell basis. Thus in HCV and ALD diseased liver, I hypothesise that BMP4 expression is elevated due to decreased per cell VEGFR-2 activation and that this may contribute to pathological processes including liver fibrosis (Fan et al., 2006), carcinogenesis (Chiu et al., 2012, Maegdefrau et al., 2009, Maegdefrau and Bosserhoff, 2012) and HCV replication.

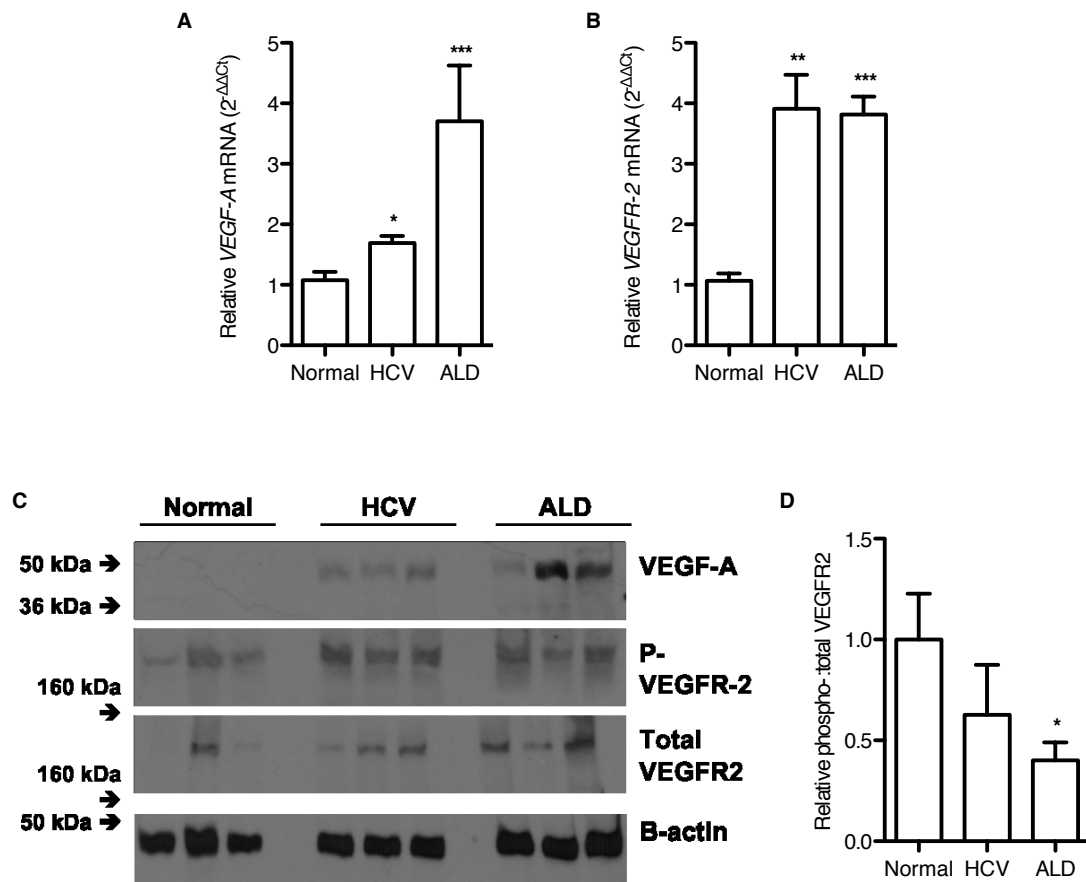


Figure 5-11. VEGFR-2 activation is reduced in chronic liver disease.

VEGF-A and VEGFR-2 expression was analysed in samples of liver tissue from normal liver, and diseased liver as a consequence of HCV infection or alcohol related liver disease (ALD). Expression of *VEGF-A* (**A**), and *VEGFR-2* (**B**) is expressed relative to normal liver control using the $2^{-\Delta\Delta C_t}$ method. Phosphorylation of VEGFR-2 is identified in whole liver (**C**) and relative activation is determined by the ratio of phospho-VEGFR-2 to total VEGFR-2 (**D**). Statistical comparisons were made with the Kruskal-Wallis test with Dunn's correction where * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, vs. normal liver control (n=6 for each liver disease type).

Finally since VEGFR-1 has been reported to act as a decoy receptor to limit VEGFR-2 activation (Fischer et al., 2008) I sought to determine expression of VEGFR-1 in liver disease. Indeed VEGFR-1 expression was induced in liver disease at comparable levels to VEGFR-2 (**Fig 5-12A**). Furthermore I was interested to understand whether VEGFR-1 was regulated in LSEC by VEGF-A stimulation. VEGF-A expression was increased in liver disease and in cultured LSEC VEGF-A stimulation significantly increased expression of *VEGFR-1* (**Fig. 5-12B**). These findings support a hypothesis where VEGFR-2 signals in endothelial cells in diseased liver may be limited by increased expression of VEGFR-1.

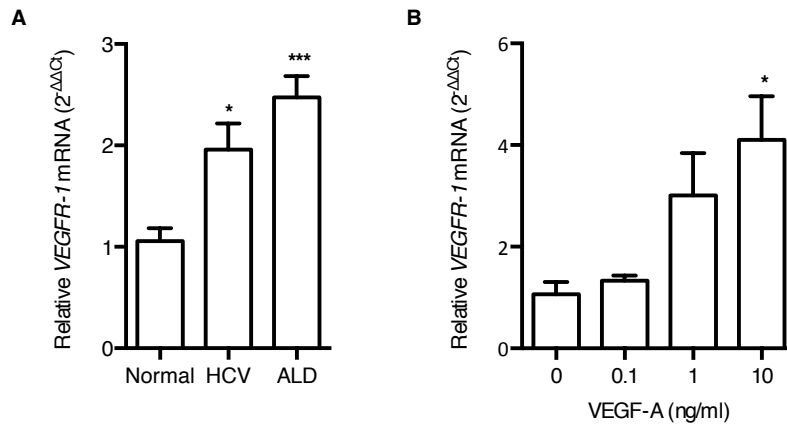


Figure 5-12. VEGFR-1 expression is regulated by VEGF-A and is increased in liver disease.

Whole liver samples (normal liver, HCV infected, and alcohol related liver disease, each $n=6$) were assessed for *VEGFR-1* expression by quantitative RT-PCR and is expressed relative to normal liver control using the $2^{-\Delta\Delta C_t}$ method (**A**). LSEC were starved of VEGF-A overnight and then stimulated with increasing doses of VEGF-A as indicated for 18 hours. *VEGFR-1* expression was analysed by quantitative RT-PCR as above (**B**). Statistical comparisons were made with the Kruskal-Wallis test with Dunn's correction where * $P<0.05$, and ** $P<0.01$, vs. untreated, or normal liver control as indicated ($n=6$ for each liver disease type).

5.6 Discussion

BMP4 is a proviral factor in HCV infection that is regulated by VEGF-A

These studies identify two novel aspects of liver pathophysiology with implications for HCV pathogenesis and liver disease in general. Firstly, I have identified BMP4 as a proviral molecule that is expressed in the chronically infected liver, and secondly I have described a role for VEGF-A in regulating endothelial BMP4 expression in the liver. The pathway defining VEGF-A regulated BMP4 expression in LSEC is illustrated in **Fig. 5-13**.

BMP4 is a member of a large group of related growth factors that constitute the largest subgroup of the TGF β superfamily (Lavery et al., 2008). These growth factors were initially identified for their ability to induce ectopic bone formation but it is increasingly recognised that many play substantial roles in development. Several members of the family are critical for early development since genetic deletion is embryonically lethal (Winnier et al., 1995, Bragdon et al., 2011). Indeed genetic deletion of BMP4 results in multiple defects during embryogenesis including failed gastrulation, mesoderm formation, and primitive haematopoiesis (Sadlon et al., 2004). BMP4 is also involved in liver development where expression in the septum transversum mesenchyme is responsible for specification of the endoderm to a liver fate (Rossi et al., 2001, Si-Tayeb et al., 2010).

Similar to other members of the TGF β family the BMPs have a common structure and signalling pathway (Bragdon et al., 2011). Mature BMP dimers bind to transmembrane type I, and type II serine threonine kinase receptors (reviewed in (Derynck and Zhang, 2003, Wharton and Derynck, 2009). BMPs can bind three different type I, and 3 different type II receptors. BMP4 binds to type I receptors BMPR-IA, BMPR-IB, and ALK-2, and to type II receptors BMPR-II, ActR-II, and ActR-IIB. Signals transduced by these receptors result in the phosphorylation of receptor-regulated R-SMAD proteins (SMAD1, SMAD5, and SMAD8). These activated SMADs form a complex with the co-SMAD (SMAD4) that translocates to the nucleus to stimulate the transcription of an array of target genes. Signals may also be transduced through non-SMAD pathways including p38 and ERK MAPKs. Thus signalling by the BMP family is complex and requires regulation to ensure correct transmission of signals. Regulation is managed in part by receptor specificity however, a number of BMP antagonists exist to regulate extracellular BMP activities. Noggin is the best characterised BMP antagonist and is known to antagonise BMP4, as well as BMP2, -5, -6, -7, -13, and -14 (Smith and Harland, 1992, Gazzerro et al., 1998, Bachiller et al., 2000). Lastly inhibitory SMADs, and SMURF ubiquitin ligases modulate intracellular signals (Massague et al., 2005) highlighting the multiple levels for regulating BMP signalling, as might be expected for processes that are critical for the early development of the organism.

Additional function(s) of BMP4 in the liver have not been investigated in detail. In the normal adult liver were observed very low levels of BMP4 were observed and expression was increased during liver injury. These findings suggest that BMP4 expression may be activated as part of reparative processes. The observation that BMP4 stimulates HCV replication is supported by two independent publications. Firstly, BMP4 is a member of the TGF β superfamily and recent reports demonstrate that TGF β can promote HCV replication (Lin et al., 2008). Secondly, in a siRNA screen of host cell factors implicated in HCV replication, Smad5 knockdown was shown to limit HCV replication further highlighting a role for these molecules in the viral lifecycle (Li et al., 2009). Thus factors expressed in the injured liver, including TGF β and BMP4, facilitate viral replication suggesting that this may be an adaptive response by HCV. Furthermore since BMP4 is expressed at low levels in the normal liver BMP4 represents a novel target for therapeutic intervention.

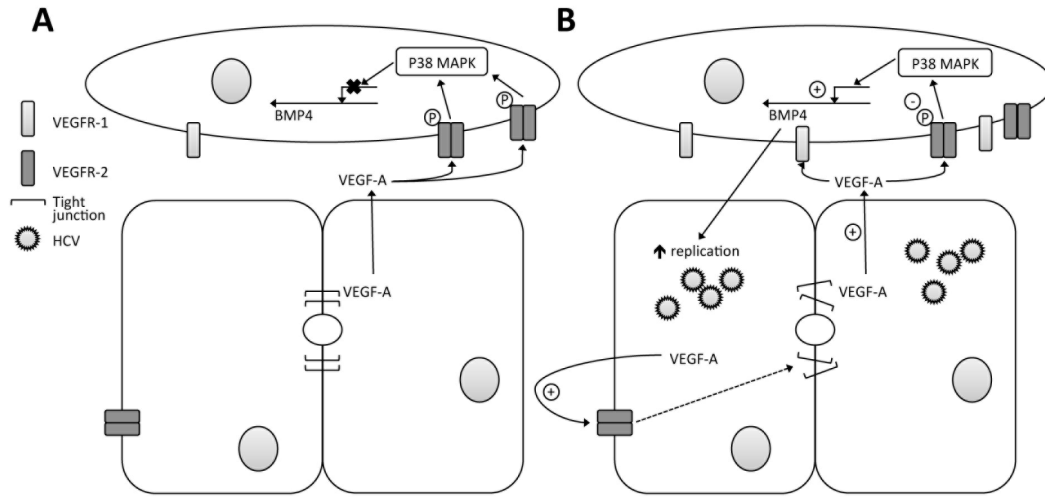


Figure 5-13. LSEC paracrine signals regulate HCV replication.

In the normal liver BMP4 expression is suppressed through VEGFR-2/p38 MAPK signalling (**A**). Following HCV infection VEGF-A expression is increased. In the hepatocyte this stimulates depolarisation and increases permissivity to HCV entry. VEGFR-2 signalling in LSEC is reduced (perhaps through increased expression of VEGFR-1) thus permitting BMP4 expression. BMP4 stimulation of hepatocytes increases HCV replication (**B**).

The role of BMP4 in the pathogenesis of chronic liver disease

The observation that BMP4 is increased in the liver of patients with advanced liver disease from different aetiologies is interesting. BMP4 was previously implicated in the development of liver fibrosis in a small animal model (Fan et al., 2006). The authors concluded that autocrine BMP4 activation of hepatic stellate cells (HSC) stimulated the development of liver fibrosis. However, my findings suggest that LSEC are a greater contributor to the expression of BMP4 than HSC. Indeed LSEC expression of BMP4 was >20 fold that in activated liver myofibroblasts (aLMF) that are representative of the activated HSC in the diseased liver. The findings suggest that a failure of VEGF-A signalling in the endothelium will drive expression of BMP4 and this in turn will activate HSC and promote liver fibrosis. A similar paracrine circuit involving VEGF-A has been described where VEGF stimulated nitric oxide maintains HSC in a quiescent phenotype (Deleve et al., 2008). Interruptions to this circuit were shown to accelerate HSC activation *in vitro*. Thus the paracrine signalling system described between hepatocytes and endothelial cells likely has further effects on additional cell types in the liver including HSC that, when perturbed, will accelerate liver fibrosis.

BMP4 has been implicated in the development and progression of multiple cancers, including HCC (Quante et al., 2011, McLean et al., 2011, Chiu et al., 2012). HCC is a major cause of mortality in patients with cirrhosis, particularly in patients with HCV infection (El-Serag, 2011). Strategies to prevent the development of HCC in the

injured liver remain one of the greatest unmet needs for patients with liver disease. Endothelial cells can maintain stem cell phenotype in the liver (Ding et al., 2010), and in other organs by expressing so-called “angiocrine” factors (Butler et al., 2010, Ding et al., 2010, Ding et al., 2011) that include BMP4 (Mathieu et al., 2008). These signals are implicated in the proliferation of epithelial cells after injury and it is likely that such factors are involved in tumorigenesis (Butler et al., 2010). Angiogenesis plays a critical role in providing oxygenated blood for tumour growth (Carmeliet and Jain, 2011). Interestingly, exogenous administration of BMP4 has been suggested as a therapeutic option for several malignancies notably glioblastoma (Piccirillo et al., 2006) and colon cancer (Lombardo et al., 2011). This option has recently been investigated in HCC (Zhang et al., 2012). Interestingly, whilst high concentration of exogenous BMP4 suppressed tumour growth, endogenous BMP4 maintained the cancer stem cell phenotype. This evidence, and my findings suggest that endothelial cells and BMP4 in particular may have a key role in driving the development of HCC in diverse forms of liver disease including HCV and ALD.

VEGF-A regulates expression of BMP4 in endothelial cells

A role for VEGF-A to regulate BMP4 expression has not previously been identified. This is a potentially important aspect of endothelial cell function since BMP4 has multiple, and pleiotropic effects in different tissues and organs. In the adult liver BMP4 is likely involved in the reparative processes following liver injury and may be

involved in the pathophysiological processes (i.e. fibrosis and carcinogenesis) that often accompany this response.

VEGF-A expression is increased in chronic liver disease (Fernandez et al., 2009) where it is associated with the inflammatory and fibrotic components of the injury. In these studies I noted an increase in both VEGF-A, and VEGF receptor expression. Stimulation of VEGFR-2 is characterised as pro-angiogenic (Quinn et al., 1993, Shalaby et al., 1995, Olsson et al., 2006), whereas VEGFR-1 acts to control the angiogenic response to VEGF-A (Fong et al., 1995, Hiratsuka et al., 1998, Fischer et al., 2008). I noted in the previous chapter that LSEC express low levels of VEGFR-1, suggesting that VEGFR-2 responses were critical for endothelial function. In whole liver VEGFR-2 activation was largely unchanged despite increased VEGF-A and VEGFR-2 expression. These changes resulted in an overall reduction in VEGFR-2 activation levels, and likely a reduction in VEGFR-2 activation on a per endothelial cell basis. Reduced VEGFR-2 activation was associated with an increase in VEGFR-1 expression. This increase may limit the activation of VEGFR-2 and permit the expression of BMP4 by endothelial cells in the liver. Notably increased VEGFR-1 expression promotes liver injury in a murine model (Mahasreshti et al., 2003). A relevant clinical correlate of this is the pre-eclampsia syndrome that is characterised by elevated soluble VEGFR-1 levels, and this too has a liver phenotype (Levine et al., 2004, Joshi et al., 2010). These observations support a role for VEGFR-1 in liver injury that may be mediated via diminishing LSEC VEGFR-2 activation.

Whilst there are clear implications of increased BMP4 expression in liver disease it is interesting to speculate on the role of this signalling system in other systems. Foremost amongst those with similar paracrine interactions is the kidney. Here there is intimate cross-talk between the renal epithelial cell, the podocyte, and the glomerular endothelial cell. The elegant work from the Quaggin group has identified the consequences of failed VEGFR-2 signalling in glomerular endothelial cells for glomerular function (Eremina et al., 2006, Eremina et al., 2008) and recent studies suggest that conditional deletion of VEGFR-2 has significant effects on sinusoidal endothelial biology (Sison et al., 2010). It is noteworthy that BMP4 signalling in the glomerulus has also been recently implicated in glomerular disease (Tominaga et al., 2011, Kishi et al., 2011) and understanding whether BMP4 is regulated by VEGF-A in glomerular endothelial cells would permit the evaluation of additional strategies to prevent the morbidity and mortality from these prevalent diseases.

In summary I have identified a role for BMP4 in the HCV lifecycle that may represent an adaptation to the reparative environment in the liver. Furthermore I have identified a critical role for VEGF-A in regulating the expression of BMP4 by endothelial cells in the liver. This paracrine signalling system has implications not only in the pathogenesis of HCV related liver injury but in liver injury of all causes. For this reason BMP4 is an attractive therapeutic target particularly since it is expressed at only very low levels in the normal liver.

Chapter 6 General Discussion

6.1 Perspective

The study of HCV has been accelerated by the discovery of first the replicon system, and subsequently HCVcc (Lindenbach et al., 2005, Wakita et al., 2005, Zhong et al., 2005). The aim of these systems has been to understand HCV biology to permit the development of rational drug therapies that will improve the outlook for patients with HCV infection.

Over the last decade there has been rapid progress in the development of directly acting antiviral (DAA) agents, particularly for genotype 1 HCV infection. Indeed the first of these agents that target the viral NS3 protease, telaprevir and boceprevir, have recently been licensed for use in this population (Zeuzem et al., 2011, Jacobson et al., 2011, Bacon et al., 2011, Poordad et al., 2011). These agents have the potential to reduce mortality from end-stage liver disease (Rowe et al., 2012), but this is associated with increased rates of adverse effects, and the risk of viral resistance (Rowe and Mutimer, 2011, Halfon and Locarnini, 2011, Pawlotsky, 2011). Further DAA agents are in development and these used in combination will likely reduce the frequency of adverse effects and increase the chance of viral eradication (Poordad and Dieterich, 2012).

Many patients however have longstanding HCV infection leading to the development of significant fibrosis, and often cirrhosis, in the liver. These patients are at risk of complications of that scarring, including the development of portal hypertension, liver failure, and the development of primary liver cancer (HCC). Whilst the probability of these events occurring after successful treatment in patients with cirrhosis is reduced, it is not zero (Singal et al., 2010b, Singal et al., 2010a). Thus there remains a significant unmet need for patients with HCV even after viral eradication.

Identification of host pathways that are implicated in the progression of liver fibrosis during liver injury, and in the development of HCC is paramount to try to address this need and improve outcomes for these patients. Following viral eradication it is likely that similar pathways that drive hepatocellular carcinogenesis will be active in both patients previously infected with HCV, and those with other liver diseases. Therefore identification of such pathways may have benefits to all patients with advanced liver disease.

6.2 LSEC are protective HCV infection

Hitherto the literature supports a role for LSEC in the capture and probable transcytosis of infectious virus particles to permissive hepatocytes (Protzer et al., 2012). However the data presented here suggest that LSEC are protective in HCV infection rather than acting to potentiate HCV as had been previously suggested. Recent data from a mouse model of adenoviral infection indicates that LSEC are responsible for scavenging the vast majority of circulating virions rapidly after inoculation (Ganesan et al., 2011). The *in vivo* studies of HCV kinetics at the time of liver transplantation support these conclusions and although the fate of these scavenged particles is not clear the *in vitro* data suggest that *trans*-infection is a rare event in HCV infection. Thus the majority of particles bound by LSEC as they enter the liver are likely processed for destruction (or antigen presentation) depleting the plasma pool of circulating virus and protecting underlying hepatocytes.

Recently available mathematical models of steady state viral kinetics supports a model where infected hepatocytes are long-lived, and new infection events are relatively rare (Ribeiro et al., 2012, Dahari et al., 2005). Since the number of particles produced per day, and the number of hepatocytes in the liver is comparable (approximately 10^{11}) (Neumann et al., 1998, Dahari et al., 2005) and survival of infected and non-infected hepatocytes is in the region of months the

majority of HCV particles are either non-infectious, or more likely destroyed in the periphery, or by scavenging LSEC during chronic infection.

The function in HCV infection of non-permissive, non-immune, cell types in the liver is a neglected area of study. Hepatocytes are protected to a large extent from toxic enteric products in portal blood by the scavenging properties of LSEC and the finding that LSEC are protective in HCV infection should not therefore come as a surprise. It is interesting however that LSEC act to reduce hepatocyte permissivity through cell contacts when these have been previously investigated for other indications. Studying these complex interactions is challenging however it would be informative to understand the changes in hepatocyte phenotype that are mediated by LSEC during HCV infection. Similar studies have been done, largely with rat hepatocytes, to dissect the mechanisms through which LSEC and hepatocyte phenotypes are maintained by this crosstalk (Bhatia et al., 1999, Khetani et al., 2004, Hui and Bhatia, 2007, March et al., 2009). Further studies employing human cells may identify further host pathways implicated in the development of liver injury in HCV infection.

6.3 The role of BMP4 in HCV infection

The hepatic lobule is a complex microenvironment and the role of (non-immune) cells other than hepatocytes in HCV infection has been well studied. I have exploited the endothelial cell hepatocyte co-culture system to identify BMP4 as a novel proviral factor that is expressed in the liver of patients with HCV infection.

The role of host factors that are upregulated in the injured liver in HCV infection has been limited to immune responses. Targeted analyses of gene expression in infected liver have identified possible contributory factors (Asselah et al., 2005, Asselah et al., 2009) but it may be difficult to discern the true effects of these factors in the cell culture models employed. Indeed these reductionist systems may give misleading results when other cell types in the liver are considered.

A prime example of this is VEGF-A. We have previously reported that this growth factor is capable of inducing hepatocyte depolarisation and increasing hepatocyte permissivity for HCV entry and infection. It was suggested that anti-VEGF therapies might have a role as an adjunctive treatment for patients with HCV infection (Himmelsbach et al., 2009, Mee et al., 2010). The work presented here suggests that treatment with anti-VEGF strategies may in fact be deleterious by activating BMP4 expression and thus accelerating viral replication. To further support these

observations in co-culture of LSEC and HepG2 cells we noted that polarised HepG2 cells were protected from VEGF-A treatment in the presence of LSEC (IAR & Christopher Mee, unpublished observation). It is true that the balance of VEGF-A signalling in the liver in HCV infection is still not completely understood. In the absence of a faithful small model of disease where normal architectural relationships are maintained and paracrine signalling is intact the community does not have the tools to dissect this further.

The identification of BMP4 as a proviral factor in HCV infection is intriguing and examining the functions of BMP4 in the developing liver (Rossi et al., 2001, Lemaigre, 2009, Si-Tayeb et al., 2010) suggests a role for BMP4 in the reparative processes that are established after infection. I therefore speculate that HCV has evolved to exploit BMP4 signalling as a primer for replication. This hypothesis is supported by the report that TGF β also increases HCV replication (Lin et al., 2008) suggesting that this response may be common to signals that are implicated in the pathogenesis of liver injury.

6.4 A role for BMP4 in liver injury and carcinogenesis

As a member of the TGF β superfamily BMP4 has been described to be involved in liver fibrosis (Fan et al., 2006, Zhong et al., 2009), and in carcinogenesis (Chiu et al., 2012, Maegdefrau et al., 2009, Maegdefrau and Bosserhoff, 2012). A central role for endothelial cell regulation of these processes has been proposed by several investigators (Deleve et al., 2008, Butler et al., 2010), and endothelial cells are critically involved in organ regeneration (Ding et al., 2011), including the liver (Ding et al., 2010, LeCouter et al., 2003). There are therefore several potential implications of VEGF-A regulation of BMP4 in endothelial cells.

BMP4 in liver fibrosis

LSEC have been described to regulate activation of hepatic stellate cells (HSC) through a VEGF-A and nitric oxide mediated mechanism (Deleve et al., 2008). This report highlights the importance of both LSEC and VEGF-A in the maintenance of normal liver architecture and also in the response to liver injury. A putative role of BMP4 in liver fibrosis has been suggested by studies in small animal models of liver fibrosis (Fan et al., 2006, Zhong et al., 2009). In the only direct evidence to date of the involvement of BMP4 in fibrosis an autocrine activation loop in HSC was proposed (Fan et al., 2006). I have shown that LSEC rather than cells of

mesenchymal origin are responsible for the majority contribution to hepatic BMP4 expression and these data suggest that LSEC may further regulate HSC function by suppressing profibrotic BMP4 in response to VEGF-A stimulation in the normal liver. Indeed I detected no change in endothelial nitric oxide synthetase activation by immunoblotting for phosphorylated protein in diseased liver (IAR, unpublished observation) suggesting a more prominent role for BMP4 in endothelial cell regulation of HSC phenotype.

BMP4 in carcinogenesis

A role for BMP4 in the development of HCC has recently been proposed (Chiu et al., 2012, Maegdefrau et al., 2009, Maegdefrau and Bosserhoff, 2012). These studies indicate that BMP4 is overexpressed in HCC, that BMP4 may be regulated by hypoxia, and that BMP4 will both stimulate hepatocellular proliferation and migration. The data highlighting increased BMP4 expression in advanced liver disease from both HCV related, and alcohol related liver injury suggest a novel mechanism through which chronic liver injury is associated with the development of HCC.

The increasing understanding of carcinogenesis supports a role for cancer stem cells in the initiation and persistence of cancer (Hanahan and Weinberg, 2011). Endothelial cells are required for maintenance of the stem cell niche in the bone

marrow (Goldman et al., 2009, Hooper et al., 2009) and the endothelial cells of the liver exhibit similar phenotypic characteristics to those of the bone marrow. It is therefore likely that LSEC have similar functions and the regulation of BMP4 by VEGF-A supports this hypothesis (Butler et al., 2010). BMP4 has been described as an “angiocrine” signal from endothelium to neural stem cells (Mathieu et al., 2008) and dysregulation of these signalling systems may provide one mechanism through which cancer progression may be accelerated.

Another intriguing possibility is the role of BMP4 in driving tumour progression in the presence of anti-VEGF treatment. Therapeutic targeting of angiogenesis was once seen as a “magic bullet” for the treatment of cancer but results have been disappointing (Bergers and Hanahan, 2008). In most cases there is a brief period of tumour control followed by inevitable progression. Indeed in HCC targeting VEGF-A through tyrosine kinase inhibition with the drug sorafenib only prolonged survival by a median of 2-3 months (Llovet et al., 2008, Cheng et al., 2009). There are multiple mechanisms described to explain these findings including a pre-existing non-reliance on VEGF-A signalling, and tumour evasion of the anti-angiogenic therapy (Bergers and Hanahan, 2008). Importantly in small animal models priming endothelium with anti-VEGF treatment before inoculation with cancer cells accelerates disease progression and the occurrence of metastatic spread highlighting a critical role for the endothelium in both of these processes (Paez-Ribes et al., 2009, Ebos et al., 2009).

The observation that VEGF-A regulates BMP4 expression suggests two additional mechanisms through which tumour progression might be accelerated in these circumstances. Firstly, BMP4 has been described to accelerate tumour progression in several models of carcinogenesis (Butler et al., 2010) and inhibiting VEGF-A will promote endothelial BMP4 expression. Secondly, BMP4 has been described to stimulate endothelial to mesenchymal transition and acquisition of stem cell-like phenotype (Medici et al., 2010). Such mesenchymal cells expressing BMP4 have been implicated in the progression of ovarian cancer (McLean et al., 2011, Quante et al., 2011) suggesting a further deleterious aspect of antiangiogenic therapy on the endothelium.

6.5 Conclusions

These studies have highlighted a role for LSEC in the protection of the liver against HCV infection and have implicated BMP4 in HCV replication and persistence. Most importantly however I have identified a novel paracrine signalling system where hepatocyte derived VEGF-A suppresses endothelial expression of BMP4. This system is dysregulated in liver disease perhaps as a reparative response to liver injury and this aspect of endothelial cell biology warrants further study.

The potentially diverse roles of endothelial cell BMP4 expression in the liver suggests therapeutic manipulation of this pathway that may yield significant benefits arresting in the progression of liver disease and the development of liver cancer.

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